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**PATENT EXTENSION
A/C PATENTS**

Exhibit B

U.S. Patent 5,712,155

and

Pending Reissue Application for Patent Based Thereon

LAW OFFICES
SUGHRUE, MION, ZINN, MACPEAK & SEAS, PLLC

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August 31, 1998

BOX PATENT APPLICATION
Assistant Commissioner
for Patents
Washington, D.C. 20231

Re: Reissue Application of
Craig A. SMITH, Raymond G. GOODWIN
and M. Patricia BECKMANN entitled
"DNA ENCODING TUMOR NECROSIS
FACTOR- α AND - β RECEPTORS"
Our Ref: A-7210

Dear Sir:

This is a request for filing a Reissue Application of U.S. Patent No. 5,712,155, which issued on January 27, 1998, by Craig A. SMITH, Raymond G. GOODWIN and M. Patricia BECKMANN entitled "DNA ENCODING TUMOR NECROSIS FACTOR- α AND - β RECEPTORS".^{1/}

This application is being filed under 37 C.F.R. § 1.171. Enclosed is the specification, claims, Abstract pursuant to 37 C.F.R. § 1.173, six (6) sheets of drawings, an executed Reissue Declaration and Power of Attorney and an Information Disclosure Statement.

Consent of the Assignee to Reissue Pursuant to 37 C.F.R. § 1.172(a), and Offer to Surrender Letters Patent Pursuant to 37 C.F.R. § 1.178, are also submitted herewith.

^{1/} A Certificate of Correction for U.S. Patent 5,712,155 was issued August 18, 1998, to correct minor printing errors.

August 31, 1998

Page 2

The application underlying U.S. Patent No. 5,712,155 was assigned to Examiner Daryl Basham in Group Art Unit 1646.

The Government filing fee is calculated as follows:

Total Claims.....	<u>271</u> - 20 = <u>251</u>	x \$22 =	<u>\$5,522.00</u>
Independent Claims..	<u>17</u> - 3 = <u>14</u>	x \$82 =	<u>\$1,148.00</u>
Base Fee			<u>\$ 790.00</u>
Multiple Dependent Fee (\$ 270.00).....			<u>\$ 270.00</u>
TOTAL FILING FEE.....			<u>\$7,730.00</u>

The Assistant Commissioner is hereby authorized to charge Applicants' Deposit Account No. 19-4880 in the amount of \$ 7,730.00 for the government fee.

The Assistant Commissioner is also hereby directed and authorized to charge or credit any difference or overpayment to Deposit Account No. 19-4880.

The Assistant Commissioner is also hereby authorized to charge any fees under 37 C.F.R. §§ 1.16 and 1.17, and any petitions for extension of time under 37 C.F.R. § 1.136, which may be required during the entire pendency of the application to Deposit Account No. 19-4880. A duplicate copy of this transmittal letter is attached.

Respectfully submitted,

SUGHRUE MION, ZINN,
MACPEAK & SEAS, PLLC


Gordon Kit

Registration No. 30,764

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Reissue Application of:

CRAIG A. SMITH et al

Reissue Application of:
U.S. Patent 5,712,155

Group Art Unit: 1646

Examiner: Basham, D.

Issued: January 27, 1998

Reissue Application Filed : August 31, 1998

For: DNA ENCODING TUMOR NECROSIS
FACTOR- α AND - β RECEPTORS

CONSENT OF THE ASSIGNEE TO REISSUE PURSUANT
TO 37 C.F.R. § 1.171(a)

Assistant Commissioner
for Patents
Washington, D.C. 20231

Sir:

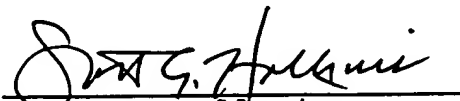
Immunex Corporation is the assignee, i.e., owner, of the entire right, title and interest of the technology disclosed and claimed in Smith et al, U.S. Patent 5,712,155, which issued on January 27, 1998, as evidenced by the Assignment recorded in Parent U.S. Patent Application Serial No. 07/523,635, at Reel 5325, Frame 0315.

Immunex Corporation hereby certifies that the above-mentioned Assignment has been reviewed and to the best of Immunex Corporation's knowledge and belief, title is in Immunex Corporation which is seeking to take this action.

Immunex Corporation hereby consents to the filing of a reissue application of Smith et al, U.S. Patent 5,712,155.

**CONSENT OF THE ASSIGNEE TO REISSUE
PURSUANT TO 37 C.F.R. § 1.171(a)
U.S. Patent No. 5,712,155**

By virtue of my position at Immunex Corporation, I am authorized to sign this written consent on behalf of the assignee, i.e., Immunex Corporation, of Smith et al, U.S. Patent 5,712,155.

A handwritten signature in dark ink, appearing to read "Scott G. Hallquist", is written over a horizontal line.

Scott G. Hallquist
Senior Vice President
General Counsel
Immunex Corporation

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Reissue Application of:

CRAIG A. SMITH et al

Reissue Application of:
U.S. Patent 5,712,155

Group Art Unit: 1646

Examiner: Basham, D.

Issued: January 27, 1998

Reissue Application Filed : August 31, 1998

For: DNA ENCODING TUMOR NECROSIS
FACTOR- α AND - β RECEPTORS

OFFER TO SURRENDER LETTERS PATENT PURSUANT
TO 37 C.F.R. § 1.178

Assistant Commissioner
for Patents
Washington, D.C. 20231

Sir:

Immunex Corporation is the assignees, i.e., owner, of the entire right, title and interest of the technology disclosed and claimed in Smith et al, U.S. Patent 5,712,155, which issued on January 27, 1998, as evidenced by the Assignments recorded in Parent U.S. Patent Application Serial No. 07/523,635, on at Reel 5325, Frame 0315.


Immunex Corporation hereby certifies that the above-mentioned Assignment has been reviewed and to the best of Immunex Corporation's knowledge and belief, title is in Immunex Corporation which is seeking to take this action.

OFFER TO SURRENDER LETTERS PATENT
PURSUANT TO 37 C.F.R. § 1.178
U.S. Patent 5,712,155

Immunex Corporation hereby offers to surrender the original Letters Patent U.S. Patent 5,712,155 to the United States Patent and Trademark Office.

By virtue of my position at Immunex Corporation, I am authorized to sign this offer to surrender on behalf of the assignee, i.e., Immunex Corporation, of Smith et al, U.S. Patent 5,712,155.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of U.S. Patent 5,712,155.


Scott G. Hallquist
Senior Vice President
General Counsel
Immunex Corporation

SOLE/JOINT

REISSUE DECLARATION AND POWER OF ATTORNEY

As below named inventors, we hereby declare that our residence, post office address and citizenship are as stated below next to our name: that we verily believe we are the original, first and joint inventors of the subject matter which is described and claimed in U.S. Patent 5,712,155, granted January 27, 1998, and for which a reissue patent is sought on the invention entitled:

**DNA ENCODING TUMOR NECROSIS
FACTOR- α AND - β RECEPTORS**

the specification of which is attached hereto.

We have reviewed and understand the contents of the above-identified specification, including the claims; that we acknowledge our duty to disclose information of which we are aware which is material to the patentability of this application under 37 C.F.R. 1.56. We verily believe the original patent to be wholly or partially inoperative or invalid by reason of the patentees claiming less than we had a right to claim, i.e., due to our Attorney's failure to appreciate the full scope of our invention, we did not initially claim a process for producing the protein capable of binding TNF (now sought in Reissue Claims 28-31, 42-45, 56-59, 70-73 and 84-87), and we did not claim the varied scope of DNA molecules, vectors and host cells (now sought in Reissue Claims 18-27, 32-41, 46-55, 60-69 and 74-83).

We hereby claim priority benefits under Title 35, United States Code §119, §172 or §365 of any provisional application or foreign application(s) for patent or inventor's certificate listed below and have also identified on said list any foreign application for patent or inventor's certificate on this invention having a filing date before that of any foreign application on which priority is claimed:

Application Number	Country	Filing Date	Priority Claimed (yes or no)
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We hereby claim the benefit of Title 35, United States Code §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in a listed prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, We acknowledge our duty to disclose any information material to the patentability of this application under 37 C.F.R. 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status
07/403,241	September 5, 1989	Abandoned
07/405,370	September 11, 1989	Abandoned
07/421,417	October 13, 1989	Abandoned
07/523,635	May 10, 1990	Patented (U.S. Patent 5,395,760)
08/346,555	November 29, 1994	Patented (U.S. Patent 5,712,155)

All errors corrected in this reissue application arose without any deception intention on the part of applicants.

We hereby appoint John H. Mion, Reg. No. 18,879; Thomas J. Macpeak, Reg. No. 19,292; Robert J. Seas, Jr., Reg. No. 21,092; Darryl Mexic, Reg. No. 23,063; Robert V. Sloan, Reg. No. 22,775; Peter D. Olexy, Reg. No. 24,513; J. Frank Osha, Reg. No. 24,625; Waddell A. Biggart, Reg. No. 24,861; Louis Gubinsky, Reg. No. 24,835; Neil B. Siegel, Reg. No. 25,200; David J. Cushing, Reg. No. 28,703; John R. Inge, Reg. No. 26,916; Joseph J. Ruch, Jr., Reg. No. 26,577; Sheldon I. Landsman, Reg. No. 25,430; Richard C. Turner, Reg. No. 29,710; Howard L. Bernstein, Reg. No. 25,665; Alan J. Kasper, Reg. No. 25,426; Kenneth J. Burchfiel, Reg. No. 31,333; Gordon Kit, Reg. No. 30,764; Susan J. Mack, Reg. No. 30,951; Frank L. Bernstein, Reg. No. 31,484; Mark Boland, Reg. No. 32,197; William H. Mandir, Reg. No. 32,156; Scott M. Danicis, Reg. No. 32,562; Brian W. Hannon, Reg. No. 32,778; Abraham J. Rosner, Reg. No. 33,276; Bruce E. Kramer, Reg. No. 33,725; Paul F. Neils, Reg. No. 33,102; Brett S. Sylvester, Reg. No. 32,765; and Robert M. Masters, Reg. No. 35,603, my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and request that all correspondence about the application be addressed to **SUGHRUE, MION, ZINN, MACPEAK & SEAS, PLLC**, 2100 Pennsylvania Avenue, N.W., Washington, D.C. 20037-3202.

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date 8/28/98 First Inventor Craig A. SMITH
First Name Middle Initial Last Name
 Residence Seattle, WASHINGTON Signature Craig A. Smith
 Post Office Address: 2405 5th West, Seattle, WASHINGTON 98119
 Citizenship U.S.A.

Date 8/28/98 Second Inventor Raymond G. GOODWIN
First Name Middle Initial Last Name
 Residence Seattle, WASHINGTON Signature Raymond G. Goodwin
 Post Office Address: 3322 8th Avenue West, Seattle, WASHINGTON 98119
 Citizenship U.S.A.

Date 8/28/98 Third Inventor M. Patricia BECKMANN
First Name Middle Initial Last Name
 Residence Poulsbo, WASHINGTON Signature Patricia Beckmann
 Post Office Address: 5454 Ragan Lane, Poulsbo, WASHINGTON 98370
 Citizenship U.S.A.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Reissue Application of:

CRAIG A. SMITH et al

Reissue Application
of: U.S. Patent 5,712,155

Group Art Unit: 1646

Issued: January 27, 1998

Examiner: Basham, D.

Reissue Application Filed: August 31, 1998

For: DNA ENCODING TUMOR NECROSIS
FACTOR- α AND - β RECEPTORS

INFORMATION DISCLOSURE STATEMENT
UNDER 37 C.F.R. §§ 1.97 and 1.98

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

In accordance with the duty of disclosure under 37 C.F.R. § 1.56, Applicants hereby notify the U.S. Patent and Trademark Office of the documents which are listed on the attached Form PTO-1449 which the Examiner may deem relevant to the patentability of the claims of the above-identified application.

The listed documents were either cited by the Examiner or brought to the Examiner's attention by Applicants in Parent U.S. Patent No. 5,712,155; Grandparent U.S. Application Serial No. 07/523,635 (now U.S. Patent 5,395,760); Great Grandparent U.S. Application Serial No. 07/421,417; Great-Great Grandparent U.S. Application Serial No. 07/405,370; and Great-Great-Great Grandparent U.S. Application Serial No. 07/403,241.

Also, several additional references have been cited in related U.S. Application Serial No. 08/038,765, filed March 19, 1993; U.S.

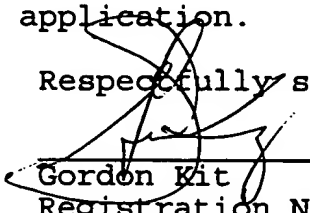
**INFORMATION DISCLOSURE STATEMENT
UNDER 37 C.F.R. §§ 1.97 and 1.98
Reissue of U.S. Patent No. 5,712,155**

Application Serial No. 08/555,629, filed November 9, 1995; and U.S. Application Serial No. 08/953,268, filed October 17, 1997, which may be considered to be relevant to patentability of the claims of the above-identified application. Hence, no references are being provided herewith.

The present Information Disclosure Statement is being filed no later than three months from the application's reissue filing date and before the mailing date of the first Office Action on the merits, and therefore no certification under 37 C.F.R. § 1.97(e) or fee under 37 C.F.R. § 1.17(p) is required.

The submission of the listed documents is not intended as an admission that any such document constitutes prior art against the claims of the present application. Applicants do not waive any right to take any action that would be appropriate to antedate or otherwise remove any listed document as a competent reference against the claims of the present application.

Respectfully submitted,



Gordon Wit
Registration No. 30,764

**SUGHRUE, MION, ZINN,
MACPEAK & SEAS, PLLC**
2100 Pennsylvania Avenue, N.W.
Washington, D.C. 20037-3202
(202) 293-7060

Date: August 31, 1998

FORM PTO-1449 U.S. DEPARTMENT OF COMMERCE
(Rev. 2-32) PATENT AND TRADEMARK OFFICE

ATTY. DOCKET NO.
A-7210

SERIAL NO.
Reissue of USP 5,715,155

INFORMATION DISCLOSURE
STATEMENT BY APPLICANT

(Use several sheets if necessary)

APPLICANT
CRAIG SMITH et al

FILING DATE
August 31, 1998

GROUP
1646

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
	5 4 7 7 8 5 1	09/05/95	Beutler et al			
	5 6 1 0 2 7 9	03/11/97	Brockhaus et al			
	5 1 1 6 9 6 4	05/26/92	Capon et al			
	5 6 0 5 6 9 0	02/25/97	Jacobs et al			
	5 1 5 5 0 2 7	10/13/93	Sledziewski et al			

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION	
						YES	NO
	4 6 4 5 3 3	06/22/91	Europe				
	4 1 7 5 6 3	03/20/91	Europe				
WO	8 9 0 2 9 2 2	04/06/89	PCT				
WO	9 1 0 8 2 9 8	06/13/91	PCT				
	0 3 9 4 8 2 7	04/19/90	Europe				
	3 2 5 2 2 4	07/26/89	Europe				

OTHER DOCUMENTS (including Author, Title, Date, Pertinent Pages, etc.)

	Ashkenazi et al, <i>Proc. Natl. Acad. Sci., USA</i> , <u>88</u> :10535-10539 (1991)
	Capon et al, <i>Nature</i> , <u>337</u> :525-530 (1989)
	Evans et al, <i>J. Exp. Med.</i> , <u>180</u> :2173-2179 (1994)
	Imamura et al, <i>J. Immunol.</i> , <u>139</u> :2989-2992 (1987)
	Ishikura et al, <i>Blood</i> , <u>73</u> :419-424 (1989)
	Jones et al, <i>Nature</i> , <u>338</u> :225-228 (1989)
	Langer et al, In: <i>New Advances on Cytokines</i> , Eds. Romagnani et al, Raven Press, New York, pages 349-354 (1992)
	Lesslauer et al, <i>Eur. J. Immunol.</i> , <u>21</u> :2883-2886 (1991)
	Loetscher et al, <i>J. Biol. Chem.</i> , <u>266</u> (2):18324-18329 (1991)
	Mohler et al, <i>J. Immunol.</i> , <u>151</u> :1548-1561 (1993)
	Peppel et al, <i>J. Cell. Biochem. Supp.</i> , <u>0(15 Part F)</u> :118 (1991)
	Peppel et al, <i>J. Exp. Med.</i> , <u>174</u> :1483-1489 (1991)
	Rutka et al, <i>Int. J. Cancer Res.</i> , <u>41</u> :573-582 (1988)
	Smith et al, <i>J. Biol. Chem.</i> , <u>262</u> :6951-6954 (1987)
	Smith et al, <i>Science</i> , <u>248</u> :1019-1023 (1990)

EXAMINER

DATE CONSIDERED

Examiner: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.
(Form PTO-1449 [6-4])

FORM PTO-1449 U.S. DEPARTMENT OF COMMERCE
(Rev. 2-32) PATENT AND TRADEMARK OFFICE

ATTY. DOCKET NO.
A-7210

SERIAL NO.
Reissue of USP 5,712,155

INFORMATION DISCLOSURE
STATEMENT BY APPLICANT

(Use several sheets if necessary)

APPLICANT
CRAIG SMITH et al

FILING DATE
August 31, 1998

GROUP
1646

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
	5 3 9 5 7 6 0	03/07/95	Smith et al			
	5 4 7 8 9 2 5	12/26/95	Wallach et al			
	4 9 3 5 2 3 3	06/19/90	Bell et al			
	5 7 1 2 1 5 5	01/27/98	Smith et al			
	5 6 9 5 9 5 3	12/09/97	Wallach et al			
	5 5 1 2 5 4 4	04/30/96	Wallach et al			
	4 6 7 5 2 8 5	06/23/87	Clark et al			

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION	
						YES	NO
	4 1 8 0 1 4	03/20/91	Europe				
	2 2 1 8 1 0 1	11/08/89	United Kingdom				
	61 2 9 3 9 2 4	12/24/86	Japan			Yes	
WO	9 0 3 5 7 5	11/15/90	PCT				

OTHER DOCUMENTS (including Author, Title, Date, Pertinent Pages, etc.)

	Dembic et al, Cytokine, 2:231-237 (1990)
	Kohno et al, Proc. Natl. Acad. Sci., USA, 87:8331-8335 (1990)
	Loetscher et al, Cell, 61:351-359 (1990)
	Nophar et al, EMBO J., 9:3269-3278 (1990)
	Pennica et al, Nature, 312:724 (1984)
	Gray et al, Nature, 312:721 (1984)
	Baglioni et al, J. Biol. Chem., 260:13395 (1985)
	Aggarwal et al, Nature, 318:665 (1985)
	Yoshie et al, J. Biochem., 100:531 (1986)
	Israel et al, Immunology Letters, 12:217 (1986)
	Creasley et al, Proc. Natl. Acad. Sci., USA, 84:3293 (1987)
	Stauber et al, J. Biol. Chem., 263(35):19098-19104 (1988)
	Aggarwal et al, J. Biol. Chem., 262:10000 (1987)
	Tsujimoto et al, J. Immun., 136:2441 (1987)
	Holtmann et al, J. Immunol., 139:1161 (1987)
	Shalaby et al, J. Leukocyte Biol., 41:196 (1987)

EXAMINER

DATE CONSIDERED

Examiner: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.
(Form PTO-1449 [6-4])

FORM PTO-1449 U.S. DEPARTMENT OF COMMERCE
(Rev. 2-32) PATENT AND TRADEMARK OFFICE

ATTY. DOCKET NO.
A-7210

SERIAL NO.
Reissue of USP 5,712,155

INFORMATION DISCLOSURE
STATEMENT BY APPLICANT

(Use several sheets if necessary)

APPLICANT
CRAIG SMITH et al

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U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION	
						YES	NO
	0 4 2 2 3 3 9	07/17/90	Europe				
	0 3 0 8 3 7 8	03/22/89	Europe				
	3 9 8 3 2 7		Europe				

OTHER DOCUMENTS (including Author, Title, Date, Pertinent Pages, etc.)

	Unglaub et al, <i>J. Exp. Med.</i> , <u>166</u> :1788 (1987)
	Yonehara et al, <i>J. Exp. Med.</i> , <u>167</u> :1511 (1988)
	Peetre et al, <i>Eur. J. Haematol.</i> , <u>41</u> :414 (1988)
	Englemann et al, <i>J. Biol. Chem.</i> , <u>264</u> :11974 (1989)
	Okayama et al, <i>Mol. Cell. Biol.</i> , <u>2</u> :161 (1982)
	Okayama et al, <i>Mol. Cell. Biol.</i> , <u>3</u> :280 (1983)
	Aruffo et al, <i>Proc. Natl. Acad. Sci., USA</i> , <u>84</u> :8573
	Yamasaki et al, <i>Science</i> , <u>241</u> :825 (1988)
	Sims et al, <i>Science</i> , <u>241</u> :585 (1988)
	Tsujimoto et al, <i>Arch. Biochem. and Biophys.</i> , pages 563-568 (1986)
	Suggs et al, <i>PNAS</i> , <u>78</u> :6613-6617 (1981)
	Kull et al, <i>PNAS</i> , <u>82</u> :5756-5760 (1985)
	Goodman, J. in <i>Basic and Clinical Immunology</i> , pages 24-25, Lange Medical Publications, Los Altos, California (1982)
	Goodman, J. in <i>Basic and Clinical Immunology</i> , pages 101-108, 7th ed., (Sites et al, eds.), Appleton & Lange, Norwalk, CONN. (1991)

EXAMINER

DATE CONSIDERED

Examiner: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.
(Form PTO-1449 (6-4))

TITLEDNA Encoding Tumor Necrosis Factor- α and - β Receptors**CROSS-REFERENCE TO RELATED APPLICATION**

This application is a Reissue of U.S. Patent No. 5,712,155, which issued from U.S. Application Serial No. 08/346,555, filed November 29, 1994; which is a Continuation of U.S. Application Serial No. 07/523,635, filed May 10, 1990, now U.S. Patent 5,395,760, which is a Continuation-In-Part of U.S. Application Serial No. 07/421,417, filed October 13, 1989[.]; now abandoned, which is a Continuation-In-Part of U.S. Application Serial No. 07/405,370, filed September 11, 1989, now abandoned, which is a Continuation-In-Part of U.S. Application Serial No. 07/403,241, filed September 5, 1989, now abandoned.

BACKGROUND OF THE INVENTION

The present invention relates generally to cytokine receptors and more specifically to tumor necrosis factor receptors.

Tumor necrosis factor- α (TNF α , also known as cachectin) and tumor necrosis factor- β (TNF β , also known as lymphotoxin) are homologous mammalian endogenous secretory proteins capable of inducing a wide variety of effects on a large number of cell types. The great similarities in the structural and functional characteristics of these two cytokines have resulted in their collective description as "TNF." Complementary cDNA clones encoding TNF α (Pennica et al., *Nature* 312:724, 1984) and TNF β (Gray et al., *Nature* 312:721, 1984) have been isolated, permitting further structural and biological characterization of TNF.

TNF proteins initiate their biological effect on cells by binding to specific TNF receptor (TNF-R) proteins expressed on the plasma membrane of a TNF-responsive cell. TNF α and TNF β were first shown to bind to a common receptor on the human cervical carcinoma cell line ME-180 (Aggarwal et al., *Nature* 318:665, 1985). Estimates of the size

of the TNF-R determined by affinity labeling studies ranged from 54 to 175 kDa (Creasey et al, *Proc. Natl. Acad. Sci. USA* 84:3293, 1987; Stauber et al., *J. Biol. Chem.* 263:19098, 1988; Hohmann et al., *J. Biol. Chem.* 264:14927, 1989). Although the relationship between these TNF-Rs of different molecular mass is unclear, Hohmann et al. (*J. Biol. Chem.* 264:14927, 1989) reported that at least two different cell surface receptors for TNF exist on different cell types. These receptors have an apparent molecular mass of about 80 kDa and about 55-60 kDa, respectively. None of the above publications, however, reported the purification to homogeneity of cell surface TNF receptors.

In addition to cell surface receptors for TNF, soluble proteins from human urine capable of binding TNF have also been identified (Peetre et al., *Eur. J. Haematol.* 41:414, 1988; Seckinger et al., *J. Exp. Med.* 167:1511, 1988; Seckinger et al., *J. Biol. Chem.* 264:11966, 1989; UK Patent Application, Publ. No. 2 218 101 A to Seckinger et al.; Engelmann et al., *J. Biol. Chem.* 264:11974, 1989). The soluble urinary TNF binding protein disclosed by UK 2 218 101 A has a partial N-terminal amino acid sequence of Asp-Ser-Val-Cys-Pro-, which corresponds to the partial sequence disclosed later by Engelmann et al. (1989). The relationship of the above soluble urinary binding proteins was further elucidated after original parent application (U.S. Serial No. 07/403,241) of the present application was filed, when Engelmann et al. reported the identification and purification of a second distinct soluble urinary TNF binding protein having an N-terminal amino acid sequence of Val-Ala-Phe-Thr-Pro- (*J. Biol. Chem.* 265:1531, 1990). The two urinary proteins disclosed by the UK 2 218 101 A and the Engelmann et al. publications were shown to be immunochemically related to two apparently distinct cell surface proteins by the ability of antiserum against the binding proteins to inhibit TNF binding to certain cells.

More recently, two separate groups reported the molecular cloning and expression of a human 55 kDa TNF-R (Loetscher et al., *Cell* 61:351, 1990; Schall et al., *Cell* 61:361, 1990). The TNF-R of both groups has an N-terminal amino acid sequence which corresponds to the partial amino acid sequence of the urinary binding protein disclosed by UK 2 218 101 A, Engelmann et al. (1989) and Engelmann et al. (1990).

In order to elucidate the relationship of the multiple forms of TNF-R and soluble urinary TNF binding proteins, or to study the structural and biological characteristics of TNF-Rs and the role played by TNF-Rs in the responses of various cell populations to TNF or other cytokine stimulation, or to use TNF-Rs effectively in therapy, diagnosis, or assay, purified compositions of TNF-R are needed. Such compositions, however, are obtainable in practical yields only by cloning and expressing genes encoding the receptors using recombinant DNA technology. [Efforst] Efforts to purify the TNF-R molecule for use in biochemical analysis or to clone and express mammalian genes encoding TNF-R, however, have been impeded by lack of a suitable source of receptor protein or mRNA. Prior to the present invention, no cell lines were known to express high levels of TNF-R constitutively and continuously, which precluded purification of receptor for sequencing or construction of genetic libraries for cDNA cloning.

SUMMARY OF THE INVENTION

The present invention provides isolated TNF receptors and DNA sequences encoding mammalian tumor necrosis factor receptors (TNF-R), in particular, human TNF-Rs. Such DNA sequences include (a) cDNA clones having a nucleotide sequence derived from the coding region of a native TNF-R gene; (b) DNA sequences which are capable of hybridization to the cDNA clones of (a) under moderately stringent conditions and which encode biologically active TNF-R molecules; or (c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode biologically active TNF-R molecules. In particular, the present invention provides DNA sequences which encode soluble TNF receptors.

The present invention also provides recombinant expression vectors comprising the DNA sequences defined above, recombinant TNF-R molecules produced using the recombinant expression vectors, and processes for producing the recombinant TNF-R molecules using the expression vectors.

The present invention also provides isolated or purified protein compositions comprising TNF-R, and, in particular, soluble forms of TNF-R.

The present invention also provides compositions for use in therapy, diagnosis, assay of TNF-R, or in raising antibodies to TNF-R, comprising effective quantities of soluble native or recombinant receptor proteins prepared according to the foregoing processes.

5 Because of the ability of TNF to specifically bind TNF receptors (TNF-Rs), purified TNF-R compositions will be useful in diagnostic assays for TNF, as well as in raising antibodies to TNF receptor for use in diagnosis and therapy. In addition, purified TNF receptor compositions may be used directly in therapy to bind or scavenge TNF, thereby providing a means for regulating the immune activities of this cytokine.

10 These and other aspects of the present invention will become evident upon reference to the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1 is a schematic representation of the coding region of various cDNAs encoding human and murine TNF-Rs. The leader sequence is hatched and the transmembrane region is solid.

20 Figures 2A-2B depict the partial cDNA sequence and derived amino acid sequence of the human TNF-R clone 1. Nucleotides are numbered from the beginning of the 5' untranslated region. Amino acids are numbered from the beginning of the signal peptide sequence. The putative signal peptide sequence is represented by the amino acids -22 to -1. The N-terminal leucine of the mature TNF-R protein is underlined at position 1. The predicted transmembrane region from amino acids 236 to 265 is also underlined. The C-termini of various soluble TNF-Rs are marked with an arrow (↑).

25 Figures 3A-3C depict the cDNA sequence and derived amino acid sequence of murine TNF-R clone 11. The putative signal peptide sequence is represented by amino acids -22 to -1. The N-terminal valine of the mature TNF-R protein is underlined at position 1. The predicted transmembrane region from amino acids 234 to 265 is also underlined.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, the terms "TNF receptor" and "TNF-R" refer to proteins having amino acid sequences which are substantially similar to the native mammalian TNF receptor amino acid sequences, and which are biologically active, as defined below, in that they are capable of binding TNF molecules or transducing a biological signal initiated by a TNF molecule binding to a cell, or cross-reacting with anti-TNF-R antibodies raised against TNF-R from natural (i.e., nonrecombinant) sources. The mature full-length human TNF-R is a glycoprotein having a molecular weight of about 80 kilodaltons (kDa). As used throughout the specification, the term "mature" means a protein expressed in a form lacking a leader sequence as may be present in full-length transcripts of a native gene. Experiments using COS cells transfected with a cDNA encoding full-length human TNF-R showed that TNF-R bound ^{125}I -TNF α with an apparent K_d of about $5 \times 10^9 \text{ M}^{-1}$, and that TNF-R bound ^{125}I -TNF β with an apparent K_d of about $2 \times 10^9 \text{ M}^{-1}$. The terms "TNF receptor" or "TNF-R" include, but are not limited to, analogs or subunits of native proteins having at least 20 amino acids and which exhibit at least some biological activity in common with TNF-R, for example, soluble TNF-R constructs which are devoid of a transmembrane region (and are secreted from the cell) but retain the ability to bind TNF. Various bioequivalent protein and amino acid analogs are described in detail below.

The nomenclature for TNF-R analogs as used herein follows the convention of naming the protein (e.g., TNF-R) preceded by either hu (for human) or mu (for murine) and followed by a Δ (to designate a deletion) and the number of the C-terminal amino acid. For example, huTNF-R Δ 235 refers to human TNF-R having Asp²³⁵ as the C-terminal amino acid (i.e., a polypeptide having the sequence of amino acids 1-235 of Figure 2A). In the absence of any human or murine species designation, TNF-R refers generically to mammalian TNF-R. Similarly, in the absence of any specific designation for deletion mutants, the term TNF-R means all forms of TNF-R, including mutants and analogs which possess TNF-R biological activity.

"Soluble TNF-R" or "sTNF-R" as used in the context of the present invention refer to proteins, or substantially equivalent analogs, having an amino acid sequence corresponding to all or part of the extracellular region of a native TNF-R, for example, huTNF-R Δ 235, huTNF-R Δ 185 and huTNF-R Δ 163, or amino acid sequences substantially similar to the sequences of amino acids 1-163, amino acids 1-185, or amino acids 1-235 of Figure 2A, and which are biologically active in that they bind to TNF ligand. Equivalent soluble TNF-Rs include polypeptides which vary from these sequences by one or more substitutions, deletions, or additions, and which retain the ability to bind TNF or inhibit TNF signal transduction activity via cell surface bound TNF receptor proteins, for example huTNF-R Δ x, wherein x is selected from the group consisting of any one of amino acids 163-235 of Figure 2A. Analogous deletions may be made to muTNF-R. Inhibition of TNF signal transduction activity can be determined by transfecting cells with recombinant TNF-R DNAs to obtain recombinant receptor expression. The cells are then contacted with TNF and the resulting metabolic effects examined. If an effect results which is attributable to the action of the ligand, then the recombinant receptor has signal transduction activity. Exemplary procedures for determining whether a polypeptide has signal transduction activity are disclosed by Idzerda et al., *J. Exp. Med.* 171:861 (1990); Curtis et al., *Proc. Natl. Acad. Sci. USA* 86:3045 (1989); Prywes et al., *EMBO J.* 5:2179 (1986) and Chou et al., *J. Biol. Chem.* 262:1842 (1987). Alternatively, primary cells or cell lines which express an endogenous TNF receptor and have a detectable biological response to TNF could also be utilized.

The term "isolated" or "purified", as used in the context of this specification to define the purity of TNF-R protein or protein compositions, means that the protein or protein composition is substantially free of other proteins of natural or endogenous origin and contains less than about 1% by mass of protein contaminants residual of production processes. Such compositions, however, can contain other proteins added as stabilizers, carriers, excipients or co-therapeutics. TNF-R is isolated if it is detectable as a single protein band in a polyacrylamide gel by silver staining.

The term "substantially similar," when used to define either amino acid or nucleic

acid sequences, means that a particular subject sequence, for example, a mutant sequence, varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which is to retain biological activity of the TNF-R protein as may be determined, for example, in one of the TNF-R binding assays set forth in Example 1
5 below. Alternatively, nucleic acid subunits and analogs are "substantially similar" to the specific DNA sequences disclosed herein if: (a) the DNA sequence is derived from the coding region of a native mammalian TNF-R gene; (b) the DNA sequence is capable of hybridization to DNA sequences of (a) under moderately stringent conditions (50°C, 2x SSC) and which encode biologically active TNF-R molecules; or DNA sequences which
10 are degenerate as a result of the genetic code to the DNA sequences defined in (a) or (b) and which encode biologically active TNF-R molecules.

"Recombinant," as used herein, means that a protein is derived from recombinant (e.g., microbial or mammalian) expression systems. "Microbial" refers to recombinant proteins made in bacterial or fungal (e.g., yeast) expression systems. As a product,
15 "recombinant microbial" defines a protein produced in a microbial expression system which is essentially free of native endogenous substances. Protein expressed in most bacterial cultures, e.g., *E. coli*, will be free of glycan. Protein expressed in yeast may have a glycosylation pattern different from that expressed in mammalian cells.

"Biologically active," as used throughout the specification as a characteristic of TNF
20 receptors, means that a particular molecule shares sufficient amino acid sequence similarity with the embodiments of the present invention disclosed herein to be capable of binding detectable quantities of TNF, transmitting a TNF stimulus to a cell, for example, as a component of a hybrid receptor construct, or cross-reacting with anti-TNF-R antibodies raised against TNF-R from natural (i.e., nonrecombinant) sources. Preferably, biologically
25 active TNF receptors within the scope of the present invention are capable of binding greater than 0.1 nmoles TNF per nmole receptor, and most preferably, greater than 0.5 nmole TNF per nmole receptor in standard binding assays (see below).

"Isolated DNA sequence" refers to a DNA polymer, in the form of a separate fragment or as a component of a larger DNA construct, which has been derived from DNA

isolated at least once in substantially pure form, i.e., free of contaminating endogenous materials and in a quantity or concentration enabling identification, manipulation, and recovery of the sequence and its component nucleotide sequences by standard biochemical methods, for example, using a cloning vector. Such sequences are preferably provided in the form of an open reading frame uninterrupted by internal nontranslated sequences, or introns, which are typically present in eukaryotic genes. Genomic DNA containing the relevant sequences could also be used as a source of coding sequences. Sequences of non-translated DNA may be present 5' or 3' from the open reading frame, where the same do not interfere with manipulation or expression of the coding regions.

"Nucleotide sequence" refers to a heteropolymer of deoxyribonucleotides. DNA sequences encoding the proteins provided by this invention can be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene which is capable of being expressed in a recombinant transcriptional unit.

Isolation of cDNAs Encoding TNF-R

The coding sequence of TNF-R is obtained by isolating a complementary DNA (cDNA) sequence encoding TNF-R from a recombinant cDNA or genomic DNA library. A cDNA library is preferably constructed by obtaining polyadenylated mRNA from a particular cell line which expresses a mammalian TNF-R, for example, the human fibroblast cell line WI-26 VA4 (ATCC CCL 95.1) and using the mRNA as a template for synthesizing double stranded cDNA. The double stranded cDNA is then packaged into a recombinant vector, which is introduced into an appropriate *E. coli* strain and propagated. Murine or other mammalian cell lines which express TNF-R may also be used. TNF-R sequences contained in the cDNA library can be readily identified by screening the library with an appropriate nucleic acid probe which is capable of hybridizing with TNF-R cDNA. Alternatively, DNAs encoding TNF-R proteins can be assembled by ligation of synthetic oligonucleotide subunits corresponding to all or part of the sequence of ~~Figures 2-3 or~~ Figures 4-6 to provide a complete coding sequence.

The human TNF receptor cDNAs of the present invention were isolated by the

method of direct expression cloning. A cDNA library was constructed by first isolating cytoplasmic mRNA from the human fibroblast cell line WI-26 VA4. Polyadenylated RNA was isolated and used to prepare double-stranded cDNA. Purified cDNA fragments were then ligated into pCAV/NOT vector DNA which uses regulatory sequences derived from pDC201 (a derivative of pMLSV, previously described by Cosman et al., *Nature* 312:768, 1984), SV40 and cytomegalovirus DNA, described in detail below in Example 2. pCAV/NOT has been deposited with the American Type Culture Collection under accession No. ATCC 68014. The pCAV/NOT vectors containing the WI26-VA4 cDNA fragments were transformed into *E. coli* strain DH5a. Transformants were plated to provide approximately 800 colonies per plate. The resulting colonies were harvested and each pool used to prepare plasmid DNA for transfection into COS-7 cells essentially as described by Cosman et al. (*Nature* 312:768, 1984) and Luthman et al. (*Nucl. Acid Res.* 11:1295, 1983). Transformants expressing biologically active cell surface TNF receptors were identified by screening for their ability to bind ¹²⁵I-TNF. In this screening approach, transfected COS-7 cells were incubated with medium containing ¹²⁵I-TNF, the cells washed to remove unbound labeled TNF, and the cell monolayers contacted with X-ray film to detect concentrations of TNF binding, as disclosed by Sims et al, *Science* 241:585 (1988). Transfectants detected in this manner appear as dark foci against a relatively light background.

Using this approach, approximately 240,000 cDNAs were screened in pools of approximately 800 cDNAs until assay of one transfectant pool indicated positive foci for TNF binding. A frozen stock of bacteria from this positive pool was grown in culture and plated to provide individual colonies, which were screened until a single clone (clone 11) was identified which was capable of directing synthesis of a surface protein with detectable TNF binding activity. The sequence of cDNA clone 11 isolated by the above method is depicted in ~~Figures 4-6.~~

Additional cDNA clones can be isolated from cDNA libraries of other mammalian species by cross-species hybridization. For use in hybridization, DNA encoding TNF-R may be covalently labeled with a detectable substance such as a fluorescent group, a

radioactive atom or a chemiluminescent group by methods well known to those skilled in the art. Such probes could also be used for *in vitro* diagnosis of particular conditions.

Like most mammalian genes, mammalian TNF receptors are presumably encoded by multi-exon genes. Alternative mRNA constructs which can be attributed to different mRNA splicing events following transcription, and which share large regions of identity or similarity with the cDNAs claimed herein, are considered to be within the scope of the present invention.

Other mammalian TNF-R cDNAs are isolated by using an appropriate human TNF-R DNA sequence as a probe for screening a particular mammalian cDNA library by cross-species hybridization.

Proteins and Analogs

The present invention provides isolated recombinant mammalian TNF-R polypeptides. Isolated TNF-R polypeptides of this invention are substantially free of other contaminating materials of natural or endogenous origin and contain less than about 1% by mass of protein contaminants residual of production processes. The native human TNF-R molecules are recovered from cell lysates as glycoproteins having an apparent molecular weight by SDS-PAGE of about 80 kilodaltons (kDa). The TNF-R polypeptides of this invention are optionally without associated native-pattern glycosylation.

Mammalian TNF-R of the present invention includes, by way of example, primate, human, murine, canine, feline, bovine, ovine, equine and porcine TNF-R. Mammalian TNF-Rs can be obtained by cross species hybridization, using a single stranded cDNA derived from the human TNF-R DNA sequence as a hybridization probe to isolate TNF-R cDNAs from mammalian cDNA libraries.

Derivatives of TNF-R within the scope of the invention also include various structural forms of the primary protein which retain biological activity. Due to the presence of ionizable amino and carboxyl groups, for example, a TNF-R protein may be in the form of acidic or basic salts, or may be in neutral form. Individual amino acid residues may also be modified by oxidation or reduction.

The primary amino acid structure may be modified by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like, or by creating amino acid sequence mutants. Covalent derivatives are prepared by linking particular functional groups to TNF-R amino acid side chains or at the N- or C-termini. Other derivatives of TNF-R within the scope of this invention include covalent or aggregative conjugates of TNF-R or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugated peptide may be a signal (or leader) polypeptide sequence at the N-terminal region of the protein which co-translationally or post-translationally directs transfer of the protein from its site of synthesis to its site of function inside or outside of the cell membrane or wall (e.g., the yeast α -factor leader). TNF-R protein fusions can comprise peptides added to facilitate purification or identification of TNF-R (e.g., poly-His). The amino acid sequence of TNF receptor can also be linked to the peptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (DYKDDDDK) (Hopp et al., *Bio/Technology* 6:1204, 1988.) The latter sequence is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein. This sequence is also specifically cleaved by bovine mucosal enterokinase at the residue immediately following the Asp-Lys pairing. Fusion proteins capped with this peptide may also be resistant to intracellular degradation in *E. coli*.

TNF-R derivatives may also be used as immunogens, reagents in receptor-based immunoassays, or as binding agents for affinity purification procedures of TNF or other binding ligands. TNF-R derivatives may also be obtained by cross-linking agents, such as M-maleimidobenzoyl succinimide ester and N-hydroxysuccinimide, at cysteine and lysine residues. TNF-R proteins may also be covalently bound through reactive side groups to various insoluble substrates, such as cyanogen bromide-activated, bisoxirane-activated, carbonyldiimidazole-activated or tosyl-activated agarose structures, or by adsorbing to polyolefin surfaces (with or without glutaraldehyde cross-linking). Once bound to a substrate, TNF-R may be used to selectively bind (for purposes of assay or

purification) anti-TNF-R antibodies or TNF.

The present invention also includes TNF-R with or without associated native-pattern glycosylation. TNF-R expressed in yeast or mammalian expression systems, e.g., COS-7 cells, may be similar or slightly different in molecular weight and glycosylation pattern than the native molecules, depending upon the expression system. Expression of TNF-R DNAs in bacteria such as *E. coli* provides non-glycosylated molecules. Functional mutant analogs of mammalian TNF-R having inactivated N-glycosylation sites can be produced by oligonucleotide synthesis and ligation or by site-specific mutagenesis techniques. These analog proteins can be produced in a homogeneous, reduced-carbohydrate form in good yield using yeast expression systems. N-glycosylation sites in eukaryotic proteins are characterized by the amino acid triplet Asn-A₁-Z, where A₁ is any amino acid except Pro, and Z is Ser or Thr. In this sequence, asparagine provides a side chain amino group for covalent attachment of carbohydrate. Such a site can be eliminated by substituting another amino acid for Asn or for residue Z, deleting Asn or Z, or inserting a non-Z amino acid between A₁ and Z, or an amino acid other than Asn between Asn and A₁.

TNF-R derivatives may also be obtained by mutations of TNF-R or its subunits. A TNF-R mutant, as referred to herein, is a polypeptide homologous to TNF-R but which has an amino acid sequence different from native TNF-R because of a deletion, insertion or substitution.

Bioequivalent analogs of TNF-R proteins may be constructed by, for example, making various substitutions of residues or sequences or deleting terminal or internal residues or sequences not needed for biological activity. For example, cysteine residues can be deleted (e.g., Cys¹⁷⁸) or replaced with other amino acids to prevent formation of unnecessary or incorrect intramolecular disulfide bridges upon renaturation. Other approaches to mutagenesis involve modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. Generally, substitutions should be made conservatively; i.e., the most preferred substitute amino acids are those having physiochemical characteristics resembling those of the residue to be replaced. Similarly, when a deletion or insertion strategy is adopted, the potential

effect of the deletion or insertion on biological activity should be considered. Substantially similar polypeptide sequences, as defined above, generally comprise a like number of amino acids sequences, although C-terminal truncations for the purpose of constructing soluble TNF-Rs will contain fewer amino acid sequences. In order to preserve the biological activity of TNF-Rs, deletions and substitutions will preferably result in homologous or conservatively substituted sequences, meaning that a given residue is replaced by a biologically similar residue. Examples of conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other such conservative substitutions, for example, substitutions of entire regions having similar hydrophobicity characteristics, are well known. Moreover, particular amino acid differences between human, murine and other mammalian TNF-Rs is suggestive of additional conservative substitutions that may be made without altering the essential biological characteristics of TNF-R.

Subunits of TNF-R may be constructed by deleting terminal or internal residues or sequences. Particularly preferred sequences include those in which the transmembrane region and intracellular domain of TNF-R are deleted or substituted with hydrophilic residues to facilitate secretion of the receptor into the cell culture medium. The resulting protein is referred to as a soluble TNF-R molecule which retains its ability to bind TNF. A particularly preferred soluble TNF-R construct is TNF-RA235 (the sequence of amino acids 1-235 of Figure-2A), which comprises the entire extracellular region of TNF-R, terminating with Asp²³⁵ immediately adjacent the transmembrane region. Additional amino acids may be deleted from the transmembrane region while retaining TNF binding activity. For example, huTNF-RA183 which comprises the sequence of amino acids 1-183 of Figure-2A, and TNF-RA163 which comprises the sequence of amino acids 1-163 of Figure-2A, retain the ability to bind TNF ligand as determined using the binding assays described below in Example 1. TNF-RA142, however, does not retain the ability to bind TNF ligand. This suggests that one or both of Cys¹⁵⁷ and Cys¹⁶³ is required for formation of an intramolecular disulfide bridge for the proper folding of TNF-R. Cys¹⁷⁸, which was

deleted without any apparent adverse effect on the ability of the soluble TNF-R to bind TNF, does not appear to be essential for proper folding of TNF-R. Thus, any deletion C-terminal to Cys¹⁶³ would be expected to result in a biologically active soluble TNF-R. The present invention contemplates such soluble TNF-R constructs corresponding to all or
5 part of the extracellular region of TNF-R terminating with any amino acid after Cys¹⁶³. Other C-terminal deletions, such as TNF- Δ 157, may be made as a matter of convenience by cutting TNF-R cDNA with appropriate restriction enzymes and, if necessary, reconstructing specific sequences with synthetic oligonucleotide linkers. The resulting soluble TNF-R constructs are then inserted and expressed in appropriate expression vectors
10 and assayed for the ability to bind TNF, as described in Example 1. Biologically active soluble TNF-Rs resulting from such constructions are also contemplated to be within the scope of the present invention.

Mutations in nucleotide sequences constructed for expression of analog TNF-R must, of course, preserve the reading frame phase of the coding sequences and preferably
15 will not create complementary regions that could hybridize to produce secondary mRNA structures such as loops or hairpins which would adversely affect translation of the receptor mRNA. Although a mutation site may be predetermined, it is not necessary that the nature of the mutation *per se* be predetermined. For example, in order to select for optimum characteristics of mutants at a given site, random mutagenesis may be conducted at the
20 target codon and the expressed TNF-R mutants screened for the desired activity.

Not all mutations in the nucleotide sequence which encodes TNF-R will be expressed in the final product, for example, nucleotide substitutions may be made to enhance expression, primarily to avoid secondary structure loops in the transcribed mRNA (see EPA 75,444A, incorporated herein by reference), or to provide codons that are more
25 readily translated by the selected host, e.g., the well-known *E. coli* preference codons for *E. coli* expression.

Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes

an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (*Gene* 42:133, 1986); Bauer et al. (*Gene* 37:73, 1985); Craik (*BioTechniques*, January 1985, 12-19); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); and U.S. Patent Nos. 4,518,584 and 4,737,462 disclose suitable techniques, and are incorporated by reference herein.

Both monovalent forms and polyvalent forms of TNF-R are useful in the compositions and methods of this invention. Polyvalent forms possess multiple TNF-R binding sites for TNF ligand. For example, a bivalent soluble TNF-R may consist of two tandem repeats of amino acids 1-235 of ~~Figure 2A~~, separated by a linker region. Alternate polyvalent forms may also be constructed, for example, by chemically coupling TNF-R to any clinically acceptable carrier molecule, a polymer selected from the group consisting of Ficoll, polyethylene glycol or dextran using conventional coupling techniques. Alternatively, TNF-R may be chemically coupled to biotin, and the biotin-TNF-R conjugate then allowed to bind to avidin, resulting in tetravalent avidin/biotin/TNF-R molecules. TNF-R may also be covalently coupled to dinitrophenol (DNP) or trinitrophenol (TNP) and the resulting conjugate precipitated with anti-DNP or anti-TNP-IgM, to form decameric conjugates with a valency of 10 for TNF-R binding sites.

A recombinant chimeric antibody molecule may also be produced having TNF-R sequences substituted for the variable domains of either or both of the [immunoglobulin] immunoglobulin molecule heavy and light chains and having unmodified constant region domains. For example, chimeric TNF-R/IgG₁ may be produced from two chimeric genes -- a TNF-R/human k light chain chimera (TNF-R/C_k) and a TNF-R/human γ_1 heavy chain chimera (TNF-R/C _{γ_1}). Following transcription and translation of the two chimeric genes, the gene products assemble into a single chimeric antibody molecule having TNF-R displayed bivalently. Such polyvalent forms of TNF-R may have enhanced binding affinity

for TNF ligand. Additional details relating to the construction of such chimeric antibody molecules are disclosed in WO 89/09622 and EP 315062.

Expression of Recombinant TNF-R

5 The present invention provides recombinant expression vectors to amplify or express DNA encoding TNF-R. Recombinant expression vectors are replicable DNA constructs which have synthetic or cDNA-derived DNA fragments encoding mammalian TNF-R or bioequivalent analogs operably linked to suitable transcriptional or translational regulatory elements derived from mammalian, microbial, viral or insect genes. A
10 transcriptional unit generally comprises an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, transcriptional promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences, as described in detail below. Such regulatory elements may include
15 an operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated. DNA regions are operably linked when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operably
20 linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of secretory leaders, contiguous and in reading frame.
25 Structural elements intended for use in yeast expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may optionally be subsequently cleaved from the expressed recombinant protein to provide a final product.

DNA sequences encoding mammalian TNF receptors which are to be expressed in a microorganism will preferably contain no introns that could prematurely terminate transcription of DNA into mRNA; however, premature termination of transcription may be desirable, for example, where it would result in mutants having advantageous C-terminal truncations, for example, deletion of a transmembrane region to yield a soluble receptor not bound to the cell membrane. Due to code degeneracy, there can be considerable variation in nucleotide sequences encoding the same amino acid sequence. Other embodiments include sequences capable of hybridizing to the sequences of the provided cDNA under moderately stringent conditions (50°C, 2x SSC) and other sequences hybridizing or degenerate to those which encode biologically active TNF receptor polypeptides.

Recombinant TNF-R DNA is expressed or amplified in a recombinant expression system comprising a substantially homogeneous monoculture of suitable host microorganisms, for example, bacteria such as *E. coli* or yeast such as *S. cerevisiae*, which have stably integrated (by transformation or transfection) a recombinant transcriptional unit into chromosomal DNA or carry the recombinant transcriptional unit as a component of a resident plasmid. Generally, cells constituting the system are the progeny of a single ancestral transformant. Recombinant expression systems as defined herein will express heterologous protein upon induction of the regulatory elements linked to the DNA sequence or synthetic gene to be expressed.

Transformed host cells are cells which have been transformed or transfected with TNF-R vectors constructed using recombinant DNA techniques. Transformed host cells ordinarily express TNF-R, but host cells transformed for purposes of cloning or amplifying TNF-R DNA do not need to express TNF-R. Expressed TNF-R will be deposited in the cell membrane or secreted into the culture supernatant, depending on the TNF-R DNA selected. Suitable host cells for expression of mammalian TNF-R include prokaryotes, yeast or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. Higher eukaryotic cells include established cell lines of mammalian origin as described below.

Cell-free translation systems could also be employed to produce mammalian TNF-R using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described by Pouwels et al. (*Cloning Vectors: A Laboratory Manual*, Elsevier, New York, 1985), the relevant disclosure of which is hereby incorporated by reference.

Prokaryotic expression hosts may be used for expression of TNF-R that do not require extensive proteolytic and disulfide processing. Prokaryotic expression vectors generally comprise one or more phenotypic selectable markers, for example a gene encoding proteins conferring antibiotic resistance or supplying an autotrophic requirement, and an origin of replication recognized by the host to ensure amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various species within the genera *Pseudomonas*, *Streptomyces*, and [*Staphylococcus*] *Staphylococcus*, although others may also be employed as a matter of choice.

Useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed. *E. coli* is typically transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species (Bolivar et al., *Gene* 2:95, 1977). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells.

Promoters commonly used in recombinant microbial expression vectors include the β -lactamase (penicillinase) and lactose promoter system (Chang et al., *Nature* 275:615, 1978; and Goeddel et al., *Nature* 281:544, 1979), the tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, 1980; and EPA 36,776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory,

p. 412, 1982). A particularly useful bacterial expression system employs the phage λ P_L promoter and cI857ts thermolabile repressor. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ P_L promoter include plasmid pHUB2, resident in *E. coli* strain JMB9 (ATCC 37092) and pPLc28, resident in *E. coli* RR1 (ATCC 53082).

Recombinant TNF-R proteins may also be expressed in yeast hosts, preferably from the *Saccharomyces* species, such as *S. cerevisiae*. Yeast of other genera, such as *Pichia* or *Kluyveromyces* may also be employed. Yeast vectors will generally contain an origin of replication from the 2 μ yeast plasmid or an autonomously replicating sequence (ARS), promoter, DNA encoding TNF-R, sequences for polyadenylation and transcription termination and a selection gene. Preferably, yeast vectors will include an origin of replication and selectable marker permitting transformation of both yeast and *E. coli*, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 or URA3 gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, and a promoter derived from a highly expressed yeast gene to induce transcription of a structural sequence downstream. The presence of the TRP1 or URA3 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan or uracil.

Suitable promoter sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149, 1968; and Holland et al., *Biochem.* 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPA 73,657.

Preferred yeast vectors can be assembled using DNA sequences from pUC18 for selection and replication in *E. coli* (Amp^r gene and origin of replication) and yeast DNA

sequences including a glucose-repressible ADH2 promoter and α -factor secretion leader. The ADH2 promoter has been described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). The yeast α -factor leader, which directs secretion of heterologous proteins, can be inserted between the promoter and the structural gene to be expressed. See, e.g., Kurjan et al., *Cell* 30:933, 1982; and Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984. The leader sequence may be modified to contain, near its 3' end, one or more useful restriction sites to facilitate fusion of the leader sequence to foreign genes.

Suitable yeast transformation protocols are known to those of skill in the art; an exemplary technique is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929, 1978, selecting for Trp⁺ transformants in a selective medium consisting of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 mg/ml adenine and 20 mg/ml uracil or URA⁺ transformants in medium consisting of 0.67% YNB, with amino acids and bases as described by Sherman et al., *Laboratory Course Manual for Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1986.

Host strains transformed by vectors comprising the ADH2 promoter may be grown for expression in a rich medium consisting of 1% yeast extract, 2% peptone, and 1% or 4% glucose supplemented with 80 mg/ml adenine and 80 mg/ml uracil. Derepression of the ADH2 promoter occurs upon exhaustion of medium glucose. Crude yeast supernatants are harvested by filtration and held at 4°C prior to further purification.

Various mammalian or insect cell culture systems are also advantageously employed to express recombinant protein. Expression of recombinant proteins in mammalian cells is particularly preferred because such proteins are generally correctly folded, appropriately modified and completely functional. Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells, described by Gluzman (*Cell* 23:175, 1981), and other cell lines capable of expressing an appropriate vector including, for example, L cells, C127, 3T3, Chinese hamster ovary (CHO), HeLa and BHK cell lines. Mammalian expression vectors may comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and

other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers,
5 *Bio/Technology* 6:47 (1988).

The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells may be provided by viral sources. For example, commonly used promoters and enhancers are derived from Polyoma, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40
10 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a heterologous DNA sequence. The early and late promoters are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., *Nature* 273:113, 1978). Smaller or larger
15 SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* 3 site toward the *Bgl*I site located in the viral origin of replication is included. Further, mammalian genomic TNF-R promoter, control and/or signal sequences may be utilized, provided such control sequences are compatible with the host cell chosen. Additional details regarding the use of a mammalian high expression vector to produce a
20 recombinant mammalian TNF receptor are provided in Examples 2 and 7 below. Exemplary vectors can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol.* 3:280, 1983).

A useful system for stable high level expression of mammalian receptor cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by
25 Cosman et al. (*Mol. Immunol.* 23:935, 1986).

In preferred aspects of the present invention, recombinant expression vectors comprising TNF-R cDNAs are stably integrated into a host cell's DNA. Elevated levels of expression product is achieved by selecting for cell lines having amplified numbers of vector DNA. Cell lines having amplified numbers of vector DNA are selected, for

example, by transforming a host cell with a vector comprising a DNA sequence which encodes an enzyme which is inhibited by a known drug. The vector may also comprise a DNA sequence which encodes a desired protein. Alternatively, the host cell may be co-transformed with a second vector which comprises the DNA sequence which encodes the desired protein. The transformed or co-transformed host cells are then cultured in increasing concentrations of the known drug, thereby selecting for drug-resistant cells. Such drug-resistant cells survive in increased concentrations of the toxic drug by over-production of the enzyme which is inhibited by the drug, frequently as a result of amplification of the gene encoding the enzyme. Where drug resistance is caused by an increase in the copy number of the vector DNA encoding the inhibitable enzyme, there is a concomitant co-amplification of the vector DNA encoding the desired protein (TNF-R) in the host cell's DNA.

A preferred system for such co-amplification uses the gene for dihydrofolate reductase (DHFR), which can be inhibited by the drug methotrexate (MTX). To achieve co-amplification, a host cell which lacks an active gene encoding DHFR is either transformed with a vector which comprises DNA sequence encoding DHFR and a desired protein, or is co-transformed with a vector comprising a DNA sequence encoding DHFR and a vector comprising a DNA sequence encoding the desired protein. The transformed or co-transformed host cells are cultured in media containing increasing levels of MTX, and those cells lines which survive are selected.

A particularly preferred co-amplification system uses the gene for glutamine synthetase (GS), which is responsible for the synthesis of glutamate and ammonia using the hydrolysis of ATP to ADP and phosphate to drive the reaction. GS is subject to inhibition by a variety of inhibitors, for example methionine sulfoximine (MSX). Thus, TNF-R can be expressed in high concentrations by co-amplifying cells transformed with a vector comprising the DNA sequence for GS and a desired protein, or co-transformed with a vector comprising a DNA sequence encoding GS and a vector comprising a DNA sequence encoding the desired protein, culturing the host cells in media containing increasing levels of MSX and selecting for surviving cells. The GS co-amplification

system, appropriate recombinant expression vectors and cells lines, are described in the following PCT applications: WO 87/04462, WO 89/01036, WO 89/10404 and WO 86/05807.

5 Recombinant proteins are preferably expressed by co-amplification of DHFR or GS in a mammalian host cell, such as Chinese Hamster Ovary (CHO) cells, or alternatively in a murine myeloma cell line, such as SP2/0-Ag14 or NS0 or a rat myeloma cell line, such as YB2/3.0-Ag20, disclosed in PCT applications WO/89/10404 and WO 86/05807.

10 A preferred eukaryotic vector for expression of TNF-R DNA is disclosed below in Example 2. This vector, referred to as pCAV/NOT, was derived from the mammalian high expression vector pDC201 and contains regulatory sequences from SV40, adenovirus-2, and human cytomegalovirus.

Purification of Recombinant TNF-R

15 Purified mammalian TNF receptors or analogs are prepared by culturing suitable host/vector systems to express the recombinant translation products of the DNAs of the present invention, which are then purified from culture media or cell extracts.

20 For example, supernatants from systems which secrete recombinant protein into culture media can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a suitable purification matrix. For example, a suitable affinity matrix can comprise a TNF or lectin or antibody molecule bound to a suitable support. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types
25 commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred.

Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant

methyl or other aliphatic groups, can be employed to further purify a TNF-R composition. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

5 Recombinant protein produced in bacterial culture is usually isolated by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography steps. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of recombinant mammalian TNF-R can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use
10 of cell lysing agents.

Fermentation of yeast which express mammalian TNF-R as a secreted protein greatly simplifies purification. Secreted recombinant protein resulting from a large-scale fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog.* 296:171, 1984). This reference describes two sequential, reversed-phase
15 HPLC steps for purification of recombinant human GM-CSF on a preparative HPLC column.

Human TNF-R synthesized in recombinant culture is characterized by the presence of non-human cell components, including proteins, in amounts and of a character which depend upon the purification steps taken to recover human TNF-R from the culture. These
20 components ordinarily will be of yeast, prokaryotic or non-human higher eukaryotic origin and preferably are present in innocuous contaminant quantities, on the order of less than about 1 percent by weight. Further, recombinant cell culture enables the production of TNF-R free of proteins which may be normally associated with TNF-R as it is found in nature in its species of origin, e.g. in cells, cell exudates or body fluids.

Therapeutic Administration of Recombinant Soluble TNF-R

25 The present invention provides methods of using therapeutic compositions comprising an effective amount of soluble TNF-R proteins and a suitable diluent and carrier, and methods for suppressing TNF-dependent inflammatory responses in humans

comprising administering an effective amount of soluble TNF-R protein.

For therapeutic use, purified soluble TNF-R protein is administered to a patient, preferably a human, for treatment in a manner appropriate to the indication. Thus, for example, soluble TNF-R protein compositions can be administered by bolus injection, continuous infusion, sustained release from implants, or other suitable technique. Typically, a soluble TNF-R therapeutic agent will be administered in the form of a composition comprising purified protein in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the TNF-R with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents. Preferably, product is formulated as a lyophilizate using appropriate excipient solutions (e.g., sucrose) as diluents. Appropriate dosages can be determined in trials. The amount and frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the condition of the patient, and so forth.

Soluble TNF-R proteins are administered for the purpose of inhibiting TNF-dependent responses. A variety of diseases or conditions are believed to be caused by TNF, such as cachexia and septic shock. In addition, other key cytokines (IL-1, IL-2 and other colony stimulating factors) can also induce significant host production of TNF. Soluble TNF-R compositions may therefore be used, for example, to treat cachexia or septic shock or to treat side effects associated with cytokine therapy. Because of the primary roles IL-1 and IL-2 play in the production of TNF, combination therapy using both IL-1 receptors or IL-2 receptors may be preferred in the treatment of TNF-associated clinical indications.

The following examples are offered by way of illustration, and not by way of

limitation.

EXAMPLES

Example 1

Binding Assays

A. *Radiolabeling of TNF α and TNF β* . Recombinant human TNF α , in the form of a fusion protein containing a hydrophilic octapeptide at the N-terminus, was expressed in yeast as a secreted protein and purified by affinity chromatography (Hopp et al., *Bio/Technology* 6:1204, 1988). Purified recombinant human TNF β was purchased from R&D Systems (Minneapolis, MN). Both proteins were radiolabeled using the commercially available solid phase agent, IODO-GEN (Pierce). In this procedure, 5 μ g of IODO-GEN were plated at the bottom of a 10 x 75 mm glass tube and incubated for 20 minutes at 4°C with 75 μ l of 0.1 M sodium phosphate, pH 7.4 and 20 μ l (2 mCi) Na ¹²⁵I. This solution was then transferred to a second glass tube containing 5 μ g TNF α (or TNF β) in 45 μ l PBS for 20 minutes at 4°C. The reaction mixture was fractionated by gel filtration on a 2 ml bed volume of Sephadex G-25 (Sigma) equilibrated in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2.5% (w/v) bovine serum albumin (BSA), 0.2% (w/v) sodium azide and 20 mM Hepes pH 7.4 (binding medium). The final pool of ¹²⁵I-TNF was diluted to a working stock solution of 1 x 10⁻⁷ M in binding medium and stored for up to one month at 4°C without detectable loss of receptor binding activity. The specific activity is routinely 1 x 10⁶ cpm/mmmole TNF.

B. *Binding to Intact Cells*. Binding assays with intact cells were performed by two methods. In the first method, cells were first grown either in suspension (e.g., U 937) or by adherence on tissue culture plates (e.g., WI26-VA4, COS cells expressing the recombinant TNF receptor). Adherent cells were subsequently removed by treatment with 5mM EDTA treatment for ten minutes at 37 degrees centigrade. Binding assays were then performed by a [phthalate] phthalate oil separation method (Dower et al., *J. Immunol.* 132:751, 1984) essentially as described by Park et al. (*J. Biol. Chem.* 261:4177, 1986). Non-specific binding of ¹²⁵I-TNF was measured in the presence of a 200-fold or greater

molar excess of unlabeled TNF. Sodium azide (0.2%) was included in a binding assay to inhibit internalization of ^{125}I -TNF by cells. In the second method, COS cells transfected with the TNF-R-containing plasmid, and expressing TNF receptors on the surface, were tested for the ability to bind ^{125}I -TNF by the plate binding assay described by Sims et al. (Science 241:585, 1988).

C. *Solid Phase Binding Assays.* The ability of TNF-R to be stably adsorbed to nitrocellulose from detergent extracts of human cells yet retain TNF-binding activity provided a means of detecting TNF-R. Cell extracts were prepared by mixing a cell pellet with a 2 x volume of PBS containing 1% Triton X-100 and a cocktail of protease inhibitors (2 mM phenylmethyl sulfonyl fluoride, 10 μM pepstatin, 10 μM leupeptin, 2 mM o-phenanthroline and 2 mM EGTA) by vigorous vortexing. The mixture was incubated on ice for 30 minutes after which it was centrifuged at 12,000x g for 15 minutes at 8°C to remove nuclei and other debris. Two microliter aliquots of cell extracts were placed on dry BA85/21 nitrocellulose membranes (Schleicher and Schuell, Keene, NH) and allowed to dry. The membranes were incubated in tissue culture dishes for 30 minutes in Tris (0.05 M) buffered saline (0.15 M) pH 7.5 containing 3% w/v BSA to block nonspecific binding sites. The membrane was then covered with 5×10^{-11} M ^{125}I -TNF in PBS + 3% BSA and incubated for 2 hr at 4°C with shaking. At the end of this time, the membranes were washed 3 times in PBS, dried and placed on Kodak X-Omat AR film for 18 hr at -70°C.

Example 2

Isolation of Human TNF-R cDNA by Direct Expression of Active Protein in COS-7

Cells

Various human cell lines were screened for expression of TNF-R based on their ability to bind ^{125}I -labeled TNF. The human fibroblast cell line WI-26 VA4 was found to express a reasonable number of receptors per cell. Equilibrium binding studies showed that the cell line exhibited biphasic binding of ^{125}I -TNF with approximately 4,000 high affinity

sites ($K_d = 1 \times 10^{10} \text{ M}^{-1}$) and 15,00 low affinity sites ($K_d = 1 \times 10^8 \text{ M}^{-1}$) per cell.

An unsized cDNA library was constructed by reverse transcription of polyadenylated mRNA isolated from total RNA extracted from human fibroblast WI-26 VA4 cells grown in the presence of pokeweed mitogen using standard techniques (Gubler, et al., *Gene* 25:263, 1983; Ausubel et al., eds., *Current Protocols in Molecular Biology*, Vol. 1, 1987). The cells were harvested by lysing the cells in a guanidine hydrochloride solution and total RNA isolated as previously described (March et al., *Nature* 315:641, 1985).

Poly A⁺ RNA was isolated by oligo dT cellulose chromatography and double-stranded cDNA was prepared by a method similar to that of Gubler and Hoffman (*Gene* 25:263, 1983). Briefly, the poly A⁺ RNA was converted to an RNA-cDNA hybrid by reverse transcriptase using oligo dT as a primer. The RNA-cDNA hybrid was then converted into double-stranded cDNA using RNAase H in combination with DNA polymerase I. The resulting double stranded cDNA was blunt-ended with T4 DNA polymerase. To the blunt-ended cDNA is added *EcoRI* linker-adapters (having internal *NotI* sites) which were phosphorylated on only one end (Invitrogen). The linker-adapted cDNA was treated with T4 polynucleotide kinase to phosphorylate the 5' overhanging region of the linker-adapter and unligated linkers were removed by running the cDNA over a Sepharose CL4B column. The linker-adapted cDNA was ligated to an equimolar concentration of *EcoRI* cut and dephosphorylated arms of bacteriophage λ gt10 (Huynh et al., *DNA Cloning: A Practical Approach*, Glover, ed., IRL Press, pp. 49-78). The ligated DNA was packaged into phage particles using a commercially available kit to generate a library of recombinants (Stratagene Cloning Systems, San Diego, CA, USA). Recombinants were further amplified by plating phage on a bacterial lawn of *E. coli* strain c600(hfl⁻).

Phage DNA was purified from the resulting λ gt10 cDNA library and the cDNA inserts excised by digestion with the restriction enzyme *NotI*. Following electrophoresis of the digest through an agarose gel, cDNAs greater than 2,000 bp were isolated.

The resulting cDNAs were ligated into the eukaryotic expression vector

pCAV/NOT, which was designed to express cDNA sequences inserted at its multiple cloning site when transfected into mammalian cells. pCAV/NOT was assembled from pDC201 (a derivative of pMLSV, previously described by Cosman et al., *Nature* 312: 768, 1984), SV40 and cytomegalovirus DNA and comprises, in sequence with the direction of transcription from the origin of replication: (1) SV40 sequences from coordinates 5171-270 including the origin of replication, enhancer sequences and early and late promoters; (2) cytomegalovirus sequences including the promoter and enhancer regions (nucleotides 671 to +63 from the sequence published by Boechart et al. (*Cell* 41:521, 1985); (3) adenovirus-2 sequences containing the first exon and part of the intron between the first and second exons of the tripartite leader, the second exon and part of the third exon of the tripartite leader and a multiple cloning site (MCS) containing sites for XhoI, KpnI, SmaI, NotI and BglI; (4) SV40 sequences from coordinates 4127-4100 and 2770-2533 that include the polyadenylation and termination signals for early transcription; (5) sequences derived from pBR322 and virus-associated sequences VAI and VAII of pDC201, with adenovirus sequences 10532-11156 containing the VAI and VAII genes, followed by pBR322 sequences from 4363-2486 and 1094-375 containing the ampicillin resistance gene and origin of replication.

The resulting WI-26 VA4 cDNA library in pCAV/NOT was used to transform *E. coli* strain DH5 α , and recombinants were plated to provide approximately 800 colonies per plate and sufficient plates to provide approximately 50,000 total colonies per screen. Colonies were scraped from each plate, pooled, and plasmid DNA prepared from each pool. The pooled DNA was then used to transfect a sub-confluent layer of monkey COS-7 cells using DEAE-dextran followed by chloroquine treatment, as described by Luthman et al. (*Nucl. Acids Res.* 11:1295, 1983) and McCutchan et al. (*J. Natl. Cancer Inst.* 41:351, 1986). The cells were then grown in culture for three days to permit transient expression of the inserted sequences. After three days, cell culture supernatants were discarded and the cell monolayers in each plate assayed for TNF binding as follows. Three ml of binding medium containing 1.2×10^{-11} M 125 I-labeled FLAG®-TNF was added to each plate and the plates incubated at 4°C for 120 minutes. This medium was then discarded, and each plate

was washed once with cold binding medium (containing no labeled TNF) and twice with cold PBS. The edges of each plate were then broken off, leaving a flat disk which was contacted with X-ray film for 72 hours at -70°C using an intensifying screen. TNF binding activity was visualized on the exposed films as a dark focus against a relatively uniform background.

After approximately 240,000 recombinants from the library had been screened in this manner, one transfectant pool was observed to provide TNF binding foci which were clearly apparent against the background exposure.

A frozen stock of bacteria from the positive pool was then used to obtain plates of approximately 150 colonies. Replicas of these plates were made on nitrocellulose filters, and the plates were then scraped and plasmid DNA prepared and transfected as described above to identify a positive plate. Bacteria from individual colonies from the nitrocellulose replica of this plate were grown in 0.2 ml cultures, which were used to obtain plasmid DNA, which was transfected into COS-7 cells as described above. In this manner, a single clone, clone 1, was isolated which was capable of inducing expression of human TNF-R in COS cells. The expression vector pCAV/NOT containing the TNF-R cDNA clone 1 has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA (Accession No. 68088) under the name pCAV/NOT-TNF-R.

Example 3

Construction of cDNAs Encoding Soluble huTNF-RA235

A cDNA encoding a soluble huTNF-RA235 (having the sequence of amino acids 1-235 of ~~Figure 2A~~) was constructed by excising an 840 bp fragment from pCAV/NOT-TNF-R with the restriction enzymes Not1 and Pvu2. Not1 cuts at the multiple cloning site of pCAV/NOT-TNF-R and Pvu2 cuts within the TNF-R coding region 20 nucleotides 5' of the transmembrane region. In order to reconstruct the 3' end of the TNF-R sequences, two oligonucleotides were synthesized and annealed to create the following oligonucleotide linker:

Pvu2 BamH1 Bgl2
 CTGAAGGGAGCACTGGCGACTAAGGATCCA
 GACTTCCCTCGTGACCGCTGATTCTAGGTCTAG
 AlaGluGlySerThrGlyAspEnd

This oligonucleotide linker has terminal Pvu2 and Bgl2 restriction sites, regenerates 20 nucleotides of the TNF-R, followed by a termination codon (underlined) and a BamH1 restriction site (for convenience in isolating the entire soluble TNF-R by Not1/BamH1 digestion). This oligonucleotide was then ligated with the 840 bp Not1/Pvu2 TNF-R insert into Bgl2/Not1 cut pCAV/NOT to yield psolhuTNF-RA235/CAVNOT, which was transfected into COS-7 cells as described above. This expression vector induced expression of soluble human TNF-R which was capable of binding TNF.

Example 4

Construction of cDNAs Encoding Soluble huTNF-RA185

A cDNA encoding a soluble huTNF-RA185 (having the sequence of amino acids 1-185 of ~~Figure 2A~~) was constructed by excising a 640 bp fragment from pCAV/NOT-TNF-R with the restriction enzymes Not1 and Bgl2. Not1 cuts at the multiple cloning site of pCAV/NO-TNF-R and Bgl2 cuts within the TNF-R coding region at nucleotide 637, which is 237 nucleotides 5' of the transmembrane region. The following oligonucleotide linkers were synthesized:

Bgl2
 5' -GATCTGTAACGTGGTGGCCATCCCTGGGAATGCAAGCATGGATGC-3'
 ACATTGCACCAACCGGTAGGGACCCCTTACGTTTCG
 IleCysAsnValValAlaIleProGlyAsnAlaSerMetAspAla

Not1
 5' - AGTCTGCACGTCCACGTCCCCCACCCTGGTGAGC -3'
 TACCTACGTCAGACGTGCAGGTGCAGGGGTGGGCCACTCGCCGG
 ValCysThrSerThrSerProThrArgEnd

The above oligonucleotide linkers reconstruct the 3' end of the receptor molecule up to nucleotide 708, followed by a termination codon (underlined). These oligonucleotides were

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Construction of cDNAs Encoding Soluble huTNF-R Δ 163

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Construction of cDNAs Encoding Soluble huTNF-R Δ 142

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was constructed as follows. First, the vector pSVLGS.1 (described in PCT Application Nos. WO87/04462 and WO89/01036, and available from Celltech, Ltd., Berkshire, UK) was cut with the BamH1 restriction enzyme and dephosphorylated with calf intestinal alkaline phosphatase (CIAP) to prevent the vector from religating to itself. The BamH1 cut pSVLGS.1 fragment was then ligated to a 2.4 kb BamH1 to Bgl2 fragment of pEE6hCMV (described in PCT Application No. WO89/01036, also available from Celltech) which was cut with Bgl2, BamH1 and Fsp1 to avoid two fragments of similar size, to yield an 11.2 kb vector designated p6/PSVLGS.1. pSVLGS.1 contains the glutamine synthetase selectable marker gene under control of the SV40 later promoter. The BamH1 to Bgl2 fragment of pEE6hCMV contains the human cytomegalovirus major immediate early promoter (hCMV), a polylinker, and the SV40 early polyadenylation signal. The coding sequences for soluble TNF-R were added to p6/PSVLGS.1 by excising a Not1 to BamH1 fragment from the expression vector psolTNFR/CAVNOT (made according to Example 3 above), blunt ending with Klenow and ligating with SmaI cut dephosphorylated p6/PSVLGS.1, thereby placing the solTNF-R coding sequences under the control of the hCMV promoter. This resulted in a single plasmid vector in which the SV40/GS and hCMB/solTNF-R transcription units are transcribed in opposite directions. This vector was designated psolTNFR/P6/PSVLGS.

psolTNFR/P6/PSVLGS was used to transfect CHO-K1 cells (available from ATCC, Rockville, MD, under accession number CCL 61) as follows. A monolayer of CHO-K1 cells were grown to subconfluency in Minimum Essential Medium (MEM) 10X (Gibco: 330-1581AJ) without glutamine and supplemented with 10% dialysed fetal bovine serum (Gibco: 220-6300AJ), 1 mM sodium pyruvate (Sigma), MEM non-essential amino acids (Gibco: 320-1140AG), 500 μ M asparagine and glutamate (Sigma) and nucleosides (30 μ M adenosine, guanosine, cytidine and uridine and 10 μ M thymidine)(Sigma).

Approximately 1×10^6 cells per 10 cm petri dish were transfected with 10 μ g of psolTNFR/P6/PSVLGS by standard calcium phosphate precipitation, substantially as described by Graham & van der Eb, *Virology* 52:456 (1983). Cells were subjected to glycerol shock (15% glycerol in serum-free culture medium for approximately 1.5 minutes)

approximately 4 hours after transfection, substantially as described by Frost & Williams, *Virology* 91:39 (1978), and then washed with serum-free medium. One day later, transfected cells were fed with fresh selective medium containing MSX at a final concentration of 25 [uM] μM. Colonies of MSX-resistant surviving cells were visible within 3-4 weeks. Surviving colonies were transferred to 24-well plates and allowed to grow to confluency in selective medium. Conditioned medium from confluent wells were then assayed for soluble TNF-R activity using the binding assay described in Example 1 above. These assays indicated that the colonies expressed biologically active soluble TNF-R.

In order to select for GS gene amplification, several MSX-resistant cell lines are transfected with psolTNFR/P6/PSVLGS and grown in various concentrations of MSX. For each cell line, approximately 1×10^6 cells are plated in gradually increasing concentrations of 100 [uM] μM, 250 [uM] μM, 500 [uM] μM and 1 mM MSX and incubated for 10-14 days. After 12 days, colonies resistant to the higher levels of MSX appear. The surviving colonies are assayed for TNF-R activity using the binding assay described above in Example 1. Each of these highly resistant cell lines contains cells which arise from multiple independent amplification events. From these cells lines, one or more of the most highly resistant cells lines are isolated. The amplified cells with high production rates are then cloned by limiting dilution cloning. Mass cell cultures of the transfectants secrete active soluble TNF-R.

Example 8

Expression of Soluble Human TNF-R in Yeast

Soluble human TNF-R was expressed in yeast with the expression vector pIXY432, which was derived from the yeast expression vector pIXY120 and plasmid pYEP352. pIXY120 is identical to pYαHuGM (ATCC 53157), except that it contains no cDNA insert and includes a polylinker/multiple cloning site with a NcoI restriction site.

A DNA fragment encoding TNF receptor and suitable for cloning into the yeast expression vector pIXY120 was first generated by polymerase chain reaction (PCR)

amplification of the extracellular portion of the full length receptor from pCAV/NOT-TNF-R (ATCC 68088). The following primers were used in this PCR amplification:

5' End Primer

5' - TTCCGGTACCTTTGGATAAAAGAGACTACAAGGAC
 Asp718->ProLeuAspLysArgAspTyrLysAsp
 GACGATGACAAGTTGCCCGCCAGGTGGCATTTACA-3'
 AspAspAspLys<----- TNF-R ----->

3' End Primer (antisense)

5' - CCCGGGATCCTTAGTCGCCAGTGCTCCCTTCAGCTGGG-3'
 BamH1>End<-----TNF-R----->

The 5' end oligonucleotide primer used in the amplification included an Asp718 restriction site at its 5' end, followed by nucleotides encoding the 3' end of the yeast a-factor leader sequence (Pro-Leu-Asp-Lys-Arg) and those encoding the 8 amino acids of the FLAG® peptide (AspTyrLysAspAspAspLys) fused to sequence encoding the 5' end of the mature receptor. The FLAG® peptide (Hopp et al., *Bio/Technology* 6:1204, 1988) is a highly antigenic sequence which reversibly binds the monoclonal antibody M1 (ATCC HB 9259). The oligonucleotide used to generate the 3' end of the PCR-derived fragment is the *antisense* strand of DNA encoding sequences which terminate the open reading frame of the receptor after nucleotide 704 of the mature coding region (following the Asp residue preceding the transmembrane domain) by introducing a TAA stop codon (underlined). The stop codon is then followed by a BamH1 restriction site. The DNA sequences encoding TNF-R are then amplified by PCR, substantially as described by Innis et al., eds., *PCR Protocols: A Guide to Methods and Applications* (Academic Press, 1990).

The PCR-derived DNA fragment encoding soluble human TNF-R was subcloned into the yeast expression vector pIXY120 by digesting the PCR-derived DNA fragment with BamH1 and Asp718 restriction enzymes, digesting pIXY120 with BamH1 and Asp718, and ligating the PCR fragment into the cut vector *in vitro* with T4 DNA ligase. The resulting construction (pIXY424) fused the open reading frame of the FLAG®-soluble

TNF receptor in-frame to the complete a-factor leader sequence and placed expression in yeast under the aegis of the regulated yeast alcohol dehydrogenase (ADH2) promoter. Identity of the nucleotide sequence of the soluble TNF receptor carried in pIXY424 with those in cDNA clone 1 were verified by DNA sequencing using the dideoxynucleotide chain termination method. pIXY424 was then transformed into *E. coli* strain RR1.

Soluble human TNF receptor was also expressed and secreted in yeast in a second vector. This second vector was generated by recovering the pIXY424 plasmid from *E. coli* and digesting with EcoR1 and BamH1 restriction enzymes to isolate the fragment spanning the region encoding the ADH2 promoter, the α -factor leader, the FLAG®-soluble TNF receptor and the stop codon. This fragment was ligated *in vitro* into EcoR1 and BamH1 cut plasmid pYEP352 (Hill et al., *Yeast* 2:163 (1986)), to yield the expression plasmid pIXY432, which was transformed into *E. coli* strain RR1.

To assess secretion of the soluble human TNF receptor from yeast, pIXY424 was purified and introduced into a diploid yeast strain of *S. cerevisiae* (XV2181) by electroporation and selection for acquisition of the plasmid-borne yeast TRP1⁺ gene on media lacking tryptophan. To assess secretion of the receptor directed by pIXY432, the plasmid was introduced into the yeast strain PB149-6b by electroporation followed by selection for the plasmid-borne URA3⁺ gene with growth on media lacking uracil. Overnight cultures were grown at 30°C in the appropriate selective media. The PB149-6b/pIXY434 transformants were diluted into YEP-1% glucose media and grown at 30°C for 38-40 hours. Supernatants were prepared by removal of cells by centrifugation, and filtration of supernatants through 0.45 μ filters.

The level of secreted receptor in the supernatants was determined by immuno-dotblot. Briefly, 1 ul of supernatants, and dilutions of the supernatants, were spotted onto nitrocellulose filters and allowed to dry. After blocking non-specific protein binding with a 3% BSA solution, the filters were incubated with diluted M1 anti-FLAG® antibody, excess antibody was removed by washing and then dilutions of horseradish peroxidase conjugated anti-mouse IgG antibodies were incubated with the filters. After removal of excess secondary antibodies, peroxidase substrates were added and color

development was allowed to proceed for approximately 10 minutes prior to removal of the substrate solution.

The anti-FLAG® reactive material found in the supernatants demonstrated that significant levels of receptor were secreted by both expression systems. Comparisons demonstrated that the pIXY432 system secreted approximately 8-16 times more soluble human TNF receptor than the pIXY424 system. The supernatants were assayed for soluble TNF-R activity, as described in Example 1, by their ability to bind ¹²⁵I-TNFα and block TNFα binding. The pIXY432 supernatants were found to contain significant levels of active soluble TNF-R.

Example 9

Isolation of Murine TNF-R cDNAs

Murine TNF-R cDNAs were isolated from a cDNA library made from murine 7B9 cells, an antigen-dependent helper T cell line derived from C57BL/6 mice, by cross-species hybridization with a human TNF-R probe. The cDNA library was constructed in λZAP (Stratagene, San Diego), substantially as described above in Example 2, by isolating polyadenylated RNA from the 7B9 cells.

A double-stranded human TNF-R cDNA probe was produced by excising an approximately 3.5 kb NotI fragment of the human TNF-R clone 1 and ³²P-labeling the cDNA using random primers (Boehringer-Mannheim).

The murine cDNA library was amplified once and a total of 900,000 plaques were screened, substantially as described in Example 2, with the human TNF-R cDNA probe. Approximately 21 positive plaques were purified, and the Bluescript plasmids containing EcoRI-linkered inserts were excised (Stratagene, San Diego). Nucleic acid sequencing of a portion of murine TNF-R clone 11 indicated that the coding sequence of the murine TNF-R was approximately 88% homologous to the corresponding nucleotide sequence of human TNF-R. A partial nucleotide sequence of murine TNF-R cDNA clone 11 is set forth in ~~Figures 3A-3B.~~

Example 10**Preparation of Monoclonal Antibodies to TNF-R**

Preparations of purified recombinant TNF-R, for example, human TNF-R, or
transfected COS cells expressing high levels of TNF-R are employed to generate
monoclonal antibodies against TNF-R using conventional techniques, for example, those
disclosed in U.S. Patent 4,411,993. Such antibodies are likely to be useful in interfering
with TNF binding to TNF receptors, for example, in ameliorating toxic or other undesired
effects of TNF, or as components of diagnostic or research assays for TNF or soluble TNF
receptor.

To immunize mice, TNF-R immunogen is emulsified in complete Freund's adjuvant
and injected in amounts ranging from 10-100 µg subcutaneously into Balb/c mice. Ten
to twelve days later, the immunized animals are boosted with additional immunogen
emulsified in incomplete Freund's adjuvant and periodically boosted thereafter on a weekly
to biweekly immunization schedule. Serum samples are periodically taken by retro-orbital
bleeding or tail-tip excision for testing by dot-blot assay (antibody sandwich) or ELISA
(enzyme-linked immunosorbent assay). Other assay procedures are also suitable.
Following detection of an appropriate antibody titer, positive animals are given an
intravenous injection of antigen in saline. Three to four days later, the animals are
sacrificed, splenocytes harvested, and fused to the murine myeloma cell line NS1.
Hybridoma cell lines generated by this procedure are plated in multiple microtiter plates
in a HAT selective medium (hypoxanthine, aminopterin, and thymidine) to inhibit
proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

Hybridoma clones thus generated can be screened by ELISA for reactivity with
TNF-R, for example, by adaptations of the techniques disclosed by Engvall et al.,
Immunochem. 8:871 (1971) and in U.S. Patent 4,703,004. Positive clones are then injected
into the peritoneal cavities of syngeneic Balb/c mice to produce ascites containing high
concentrations (>1 mg/ml) of anti-TNF-R monoclonal antibody. The resulting monoclonal
antibody can be purified by ammonium sulfate precipitation followed by gel exclusion

chromatography, and/or affinity chromatography based on binding of antibody to Protein A of *Staphylococcus aureus*.

What is claimed is:

Claim 1. An isolated DNA sequence selected from the group consisting of:

- (a) a DNA sequence that encodes a polypeptide having the amino acid sequence selected from the group consisting of amino acids 1 to X of FIG. 2A and amino acids 1 to 233 of FIG. 3A, wherein X is an amino acid from 163 to 235; and
- (b) a DNA sequence capable of hybridization to the complement of the DNA sequence of (a) under moderately stringent conditions (50°C., 2x SSC) and which encodes a polypeptide that is capable of binding to TNF and which is at least 88% identical to a polypeptide encoded by the DNA of (a).

Claim 2. An isolated DNA sequence selected from the group consisting of:

- (a) a DNA sequence that encodes a polypeptide having the amino acid sequence selected from the group consisting of amino acids 1 to X of FIG. 2A and amino acids 1 to 233 of FIG. 3A, wherein X is an amino acid from 163 to 235; and
- (b) a DNA sequence capable of hybridization to the complement of the DNA sequence of (a) under moderately stringent conditions (50°C., 2x SSC) and which encodes TNF-R protein that is capable of binding greater than 0.1 moles TNF per nmole TNF-R and which is at least 88% identical to a polypeptide encoded by the DNA of (a).

Claim 3. An isolated DNA sequence selected from the group consisting of:

- (a) a DNA sequence that encodes a polypeptide having the amino acid sequence selected from the group consisting of amino acids 1 to X of FIG. 2A and amino acids 1 to 233 of FIG. 3A, wherein X is an amino acid from 163 to 235; and

- (b) a DNA sequence capable of hybridization to the complement of the DNA sequence of (a) under moderately stringent conditions (50°C., 2x SSC) and which encodes TNF-R protein that is capable of binding greater than 0.5 nmoles TNF per nmole TNF-R and which is at least 88% identical to a polypeptide encoded by the DNA of (a).

Claim 4. A recombinant expression vector comprising the DNA sequence according to claim 1.

Claim 5. A recombinant expression vector comprising the DNA sequence according to claim 2.

Claim 6. A recombinant expression vector comprising the DNA sequence according to claim 3.

Claim 7. A host cell transformed or transfected with the vector according to claim 4.

Claim 8. A host cell transformed or transfected with the vector according to claim 5.

Claim 9. A host cell transformed or transfected with the vector according to claim 6.

Claim 10. An isolated DNA sequence selected from the group consisting of:

- (a) a DNA sequence that encodes a polypeptide having the amino acid sequence selected from the group consisting of amino acids 1 to X of FIG. 2A and amino acids 1 to 233 of FIG. 3A, wherein X is an amino acid from 163 to 235; and
- (b) a DNA sequence that encodes a polypeptide identical to the polypeptide encoded by the DNA of (a) except for modification(s) to the amino acid sequence selected from the group consisting of:
 - (i) inactivated N-linked glycosylation sites; (ii) altered KEX2 protease cleavage sites; (iii) conservative amino acid substitutions; (iv) substitution or deletion of cysteine residues; and

(v) combinations of modifications (i)-(iv); which such polypeptide is capable of binding TNF.

Claim 11. An isolated DNA sequence selected from the group consisting of:

- (a) a DNA sequence that encodes a polypeptide having the amino acid sequence selected from the group consisting of amino acids 1 to X of FIG. 2A and amino acids 1 to 233 of FIG. 3A, wherein X is an amino acid from 163 to 235; and
- (b) a DNA sequence that encodes a polypeptide identical to the polypeptide encoded by the DNA of (a) except for modification(s) to the amino acid sequence selected from the group consisting of:
 - (i) inactivated N-linked glycosylation sites; (ii) altered KEX2 protease cleavage sites; (iii) conservative amino acid substitutions; (iv) substitution or deletion of cysteine residues; and
 - (v) combinations of modifications (i)-(iv); which encoded polypeptide is capable of binding greater than 0.1 moles TNF per nmole of such polypeptide.

Claim 12. An isolated DNA sequence selected from the group consisting of:

- (a) a DNA sequence that encodes a polypeptide having the amino acid sequence selected from the group consisting of amino acids 1 to X of FIG. 2A and amino acids 1 to 233 of FIG. 3A, wherein X is an amino acid from 163 to 235; and
- (b) a DNA sequence that encodes a polypeptide identical to the polypeptide encoded by the DNA of (a) except for modification(s) to the amino acid sequence selected from the group consisting of:
 - (i) inactivated N-linked glycosylation sites; (ii) altered KEX2 protease cleavage sites; (iii) conservative amino acid substitutions; (iv) substitution or deletion of cysteine residues; and
 - (v) combinations of modifications (i)-(iv); which encoded

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polypeptide is capable of binding greater than 0.5 moles TNF per nmole of such polypeptide.

Claim 13. A recombinant expression vector comprising the DNA according to any one of claims 10, 11 or 12.

Claim 14. A host cell transformed or transfected with the vector according to claim 13.

Claim 15. A DNA sequence that encodes a polypeptide having the amino acid sequence selected from the group consisting of (a) amino acids 1-235 of FIG. 2A; and (b) a DNA sequence capable of hybridization to the DNA sequence of (a) under moderately stringent conditions (50°C., 2x SSC) and which encodes a polypeptide that is capable of binding to TNF and which is at least 88% identical to a polypeptide encoded by the DNA of (a).

Claim 16. A recombinant expression vector comprising the DNA sequence according to claim 15.

Claim 17. A host cell transformed or transfected with the vector according to claim 16.

Claim 18. An isolated DNA molecule encoding a protein comprising a sequence of amino acids selected from the group consisting of amino acids 1-163 of FIG. 2A and amino acids 1-233 of FIG. 3A, wherein said protein is capable of binding TNF.

Claim 19. The isolated DNA molecule according to Claim 18, wherein said protein comprises amino acids 1-163 of FIG. 2A.

Claim 20. The isolated DNA molecule according to Claim 18, wherein said protein comprises amino acids 1-185 of FIG. 2A.

Claim 21. The isolated DNA molecule according to Claim 18, wherein said protein comprises amino acids 1-235 of FIG. 2A.

Claim 22. An isolated DNA molecule encoding a protein selected from the group consisting of:

- (a) a polypeptide having a sequence of amino acids comprising amino acids 1-163 of FIG. 2A;

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- (b) a polypeptide having a sequence of amino acids comprising amino acids 1-233 of FIG. 3A; and
- (c) a polypeptide identical to the polypeptides of (a) or (b) except for one or more modification(s) to the sequence of amino acids selected from the group consisting of: (i) inactivated N-linked glycosylation sites; (ii) altered KEX2 protease cleavage sites; and (iii) substitution or deletion of cysteine residues,

wherein said protein is capable of binding TNF.

Claim 23. A recombinant expression vector comprising the DNA molecule according to Claim 18, 19, 20, 21 or 22.

Claim 24. A host cell transformed or transfected with the recombinant expression vector according to Claim 23.

Claim 25. The host cell of Claim 24, wherein said host cell is selected from the group consisting of a microbial cell and a mammalian cell.

Claim 26. The host cell of Claim 25, wherein said mammalian cell is selected from the group consisting of L cells, C127 cells, 3T3 cells, CHO cells, BHK cells and COS-7 cells.

Claim 27. The host cell of Claim 26, wherein said mammalian cell is CHO cells.

Claim 28. A process for producing a protein capable of binding TNF, said process comprising culturing a host cell of Claim 24 under conditions suitable to effect expression of said protein.

Claim 29. The process of Claim 28, wherein said host cell is selected from the group consisting of a microbial cell and a mammalian cell.

Claim 30. The process of Claim 29, wherein said mammalian cell is selected from the group consisting of L cells, C127 cells, 3T3 cells, CHO cells, BHK cells and COS-7 cells.

Claim 31. The process of Claim 30, wherein said mammalian cell is CHO cells.

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Claim 32. An isolated DNA molecule encoding a soluble TNF receptor protein comprising a sequence of amino acids selected from the group consisting of from about amino acid 1 to about amino acid 163 of FIG. 2A and from about amino acid 1 to about amino acid 233 of FIG. 3A, wherein said soluble TNF receptor protein is capable of binding TNF protein.

Claim 33. The isolated DNA molecule according to Claim 32, wherein said soluble TNF receptor protein comprises from about amino acid 1 to about amino acid 163 of FIG. 2A.

Claim 34. The isolated DNA molecule according to Claim 32, wherein said soluble TNF receptor protein comprises from about amino acid 1 to about amino acid 185 of FIG. 2A.

Claim 35. The isolated DNA molecule according to Claim 32, wherein said TNF soluble receptor protein comprises from about amino acid 1 to about amino acid 235 of FIG. 2A.

Claim 36. An isolated DNA molecule encoding a soluble TNF receptor protein selected from the group consisting of:

- (a) a TNF receptor polypeptide having a sequence of amino acids comprising from about amino acid 1 to about amino acid 163 of FIG. 2A;
- (b) a TNF receptor polypeptide having a sequence of amino acids comprising from about amino acid 1 to about amino acid 233 of FIG. 3A; and
- (c) a TNF receptor polypeptide identical to the TNF receptor polypeptides of (a) or (b) except for one or more modification(s) to the sequence of amino acids selected from the group consisting of: (i) inactivated N-linked glycosylation sites; (ii) altered KEX2 protease cleavage sites; and (iii) substitution or deletion of cysteine residues,

wherein said soluble TNF receptor protein is capable of binding TNF.

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Claim 37. A recombinant expression vector comprising the DNA molecule according to Claim 32, 33, 34, 35 or 36.

Claim 38. A host cell transformed or transfected with the recombinant expression vector according to Claim 37.

Claim 39. The host cell of Claim 38, wherein said host cell is selected from the group consisting of a microbial cell and a mammalian cell.

Claim 40. The host cell of Claim 39, wherein said mammalian cell is selected from the group consisting of L cells, C127 cells, 3T3 cells, CHO cells, BHK cells and COS-7 cells.

Claim 41. The host cell of Claim 40, wherein said mammalian cell is CHO cells.

Claim 42. A process for producing a protein capable of binding TNF, said process comprising culturing a host cell of Claim 38 under conditions suitable to effect expression of said protein.

Claim 43. The process of Claim 42, wherein said host cell is selected from the group consisting of a microbial cell and a mammalian cell.

Claim 44. The process of Claim 43, wherein said mammalian cell is selected from the group consisting of L cells, C127 cells, 3T3 cells, CHO cells, BHK cells and COS-7 cells.

Claim 45. The process of Claim 44, wherein said mammalian cell is CHO cells.

Claim 46. An isolated DNA molecule encoding a soluble TNF receptor protein comprising a sequence of amino acids selected from the group consisting of from amino acid 1 to amino acid 163 of FIG. 2A and from amino acid 1 to amino acid 233 of FIG. 3A, wherein said soluble TNF receptor protein is capable of binding TNF protein.

Claim 47. The isolated DNA molecule according to Claim 46, wherein said soluble TNF receptor protein comprises from amino acid 1 to amino acid 163 of FIG. 2A.

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Claim 48. The isolated DNA molecule according to Claim 46, wherein said soluble TNF receptor protein comprises from amino acid 1 to amino acid 185 of FIG. 2A.

Claim 49. The isolated DNA molecule according to Claim 46, wherein said soluble TNF receptor protein comprises from amino acid 1 to amino acid 235 of FIG. 2A.

Claim 50. An isolated DNA molecule encoding a soluble TNF receptor protein selected from the group consisting of:

- (a) a TNF receptor polypeptide having a sequence of amino acids comprising from amino acid 1 to amino acid 163 of FIG. 2A;
- (b) a TNF receptor polypeptide having a sequence of amino acids comprising from amino acid 1 to amino acid 233 of FIG. 3A;
and
- (c) a TNF receptor polypeptide identical to the TNF receptor polypeptides of (a) or (b) except for one or more modification(s) to the sequence of amino acids selected from the group consisting of: (i) inactivated N-linked glycosylation sites; (ii) altered KEX2 protease cleavage sites; and (iii) substitution or deletion of cysteine residues.

wherein said soluble TNF receptor protein is capable of binding TNF.

Claim 51. A recombinant expression vector comprising the DNA molecule according to Claim 46, 47, 48, 49 or 50.

Claim 52. A host cell transformed or transfected with the recombinant expression vector according to Claim 51.

Claim 53. The host cell of Claim 52, wherein said host cell is selected from the group consisting of a microbial cell and a mammalian cell.

Claim 54. The host cell of Claim 53, wherein said mammalian cell is selected from the group consisting of L cells, C127 cells, 3T3 cells, CHO cells, BHK cells and COS-7 cells.

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Claim 55. The host cell of Claim 54, wherein said mammalian cell is CHO cells.

Claim 56. A process for producing a protein capable of binding TNF, said process comprising culturing a host cell of Claim 52 under conditions suitable to effect expression of said protein.

Claim 57. The process of Claim 56, wherein said host cell is selected from the group consisting of a microbial cell and a mammalian cell.

Claim 58. The process of Claim 57, wherein said mammalian cell is selected from the group consisting of L cells, C127 cells, 3T3 cells, CHO cells, BHK cells and COS-7 cells.

Claim 59. The process of Claim 58, wherein said mammalian cell is CHO cells.

Claim 60. An isolated DNA molecule encoding a protein comprising a sequence of amino acids selected from the group consisting of amino acids 1-163 of FIG. 2A and amino acids 1-233 of FIG. 3A, wherein said protein lacks amino acids 236-265 of FIG. 2A and amino acids 234-265 of FIG. 3A, respectively, and wherein said protein is capable of binding TNF.

Claim 61. The isolated DNA molecule according to Claim 60, wherein said protein comprises amino acids 1-163 of FIG. 2A.

Claim 62. The isolated DNA molecule according to Claim 60, wherein said protein comprises amino acids 1-185 of FIG. 2A.

Claim 63. The isolated DNA molecule according to Claim 60, wherein said protein comprises amino acids 1-235 of FIG. 2A.

Claim 64. An isolated DNA molecule encoding a protein selected from the group consisting of:

- (a) a TNF receptor polypeptide having a sequence of amino acids comprising amino acids 1-163 of FIG. 2A, wherein said polypeptide lacks amino acids 236-265 of FIG. 2A;
- (b) a TNF receptor polypeptide having a sequence of amino acids comprising amino acids 1-233 of FIG. 3A, wherein said

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- polypeptide lacks amino acids 234-265 of FIG. 3A; and
- (c) a TNF receptor polypeptide identical to the TNF receptor polypeptides of (a) or (b) except for one or more modification(s) to the sequence of amino acids selected from the group consisting of: (i) inactivated N-linked glycosylation sites; (ii) altered KEX2 protease cleavage sites; and (iii) substitution or deletion of cysteine residues,

wherein said protein is capable of binding TNF.

Claim 65. A recombinant expression vector comprising the DNA molecule according to Claim 60, 61, 62, 63 or 64.

Claim 66. A host cell transformed or transfected with the recombinant expression vector according to Claim 65.

Claim 67. The host cell of Claim 66, wherein said host cell is selected from the group consisting of a microbial cell and a mammalian cell.

Claim 68. The host cell of Claim 67, wherein said mammalian cell is selected from the group consisting of L cells, C127 cells, 3T3 cells, CHO cells, BHK cells and COS-7 cells.

Claim 69. The host cell of Claim 68, wherein said mammalian cell is CHO cells.

Claim 70. A process for producing a protein capable of binding TNF, said process comprising culturing a host cell of Claim 67 under conditions suitable to effect expression of said protein.

Claim 71. The process of Claim 70, wherein said host cell is selected from the group consisting of a microbial cell and a mammalian cell.

Claim 72. The process of Claim 71, wherein said mammalian cell is selected from the group consisting of L cells, C127 cells, 3T3 cells, CHO cells, BHK cells and COS-7 cells.

Claim 73. The process of Claim 72, wherein said mammalian cell is CHO cells.

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Claim 74. An isolated DNA molecule encoding a protein comprising a sequence of amino acids selected from the group consisting of amino acids 1-163 of FIG. 2A and amino acids 1-233 of FIG. 3A, wherein said protein lacks a functional transmembrane region, and wherein said protein is capable of binding TNF.

Claim 75. The isolated DNA molecule according to Claim 74, wherein said protein comprises amino acids 1-163 of FIG. 2A.

Claim 76. The isolated DNA molecule according to Claim 74, wherein said protein comprises amino acids 1-185 of FIG. 2A.

Claim 77. The isolated DNA molecule according to Claim 74, wherein said protein comprises amino acids 1-235 of FIG. 2A.

Claim 78. An isolated DNA molecule encoding a protein selected from the group consisting of:

- (a) a TNF receptor polypeptide having a sequence of amino acids comprising amino acids 1-163 of FIG. 2A;
- (b) a TNF receptor polypeptide having a sequence of amino acids comprising amino acids 1-233 of FIG. 3A; and
- (c) a TNF receptor polypeptide identical to the TNF receptor polypeptides of (a) or (b) except for one or more modification(s) to the sequence of amino acids selected from the group consisting of: (i) inactivated N-linked glycosylation sites; (ii) altered KEX2 protease cleavage sites; and (iii) substitution or deletion of cysteine residues.

wherein said protein lacks a functional transmembrane region; and wherein said protein is capable of binding TNF.

Claim 79. A recombinant expression vector comprising the DNA molecule according to Claim 74, 75, 76, 77 or 78.

Claim 80. A host cell transformed or transfected with the recombinant expression vector according to Claim 79.

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Claim 81. The host cell of Claim 80, wherein said host cell is selected from the group consisting of a microbial cell and a mammalian cell.

Claim 82. The host cell of Claim 81, wherein said mammalian cell is selected from the group consisting of L cells, C127 cells, 3T3 cells, CHO cells, BHK cells and COS-7 cells.

Claim 83. The host cell of Claim 82, wherein said mammalian cell is CHO cells.

Claim 84. A process for producing a protein capable of binding TNF, said process comprising culturing a host cell of Claim 80 under conditions suitable to effect expression of said protein.

Claim 85. The process of Claim 84, wherein said host cell is selected from the group consisting of a microbial cell and a mammalian cell.

Claim 86. The process of Claim 85, wherein said mammalian cell is selected from the group consisting of L cells, C127 cells, 3T3 cells, CHO cells, BHK cells and COS-7 cells.

Claim 87. The process of Claim 86, wherein said mammalian cell is CHO cells.

ABSTRACT OF THE DISCLOSURE

Tumor necrosis factor receptor DNAs and expression vectors encoding TNF receptors, and processes for producing TNF receptors as products of recombinant cell culture, are disclosed.



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0242/0929

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**NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS
CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES**

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 CFR 1.821 - 1.825 for the following reason(s):

- ☒ 1. This application fails to comply with the requirements of 37 CFR 1.821 - 1.825.
- ☐ 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 CFR 1.821(c).
- ☐ 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 CFR 1.821(e).
- ☐ 4. A copy of the "Sequence Listing" in computer readable form has been submitted. The content of the computer readable form, however, does not comply with the requirements of 37 CFR 1.822 and/or 1.832, as indicated on the attached marked-up copy of the "Raw Sequence Listing."
- ☐ 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A substitute computer readable form must be submitted as required by 37 CFR 1.825(d).
- ☐ 6. The paper copy of the "Sequence Listing" is not the same as the computer readable form of the "Sequence Listing" as required by 37 CFR 1.821(e).
- ☐ 7. OTHER: _____

APPLICANT MUST PROVIDE:

- ☒ An initial or substitute computer readable form (CRF) copy of the "Sequence Listing."
- ☒ An initial or substitute paper copy of the "Sequence Listing," as well as an amendment directing its entry into the specification.
- ☒ A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b) or 1.825(d).

FOR QUESTIONS REGARDING COMPLIANCE WITH THESE REQUIREMENTS, PLEASE CONTACT:

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R. Rawls

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PART 1 - ATTORNEY/APPLICANT COPY

Figure 1

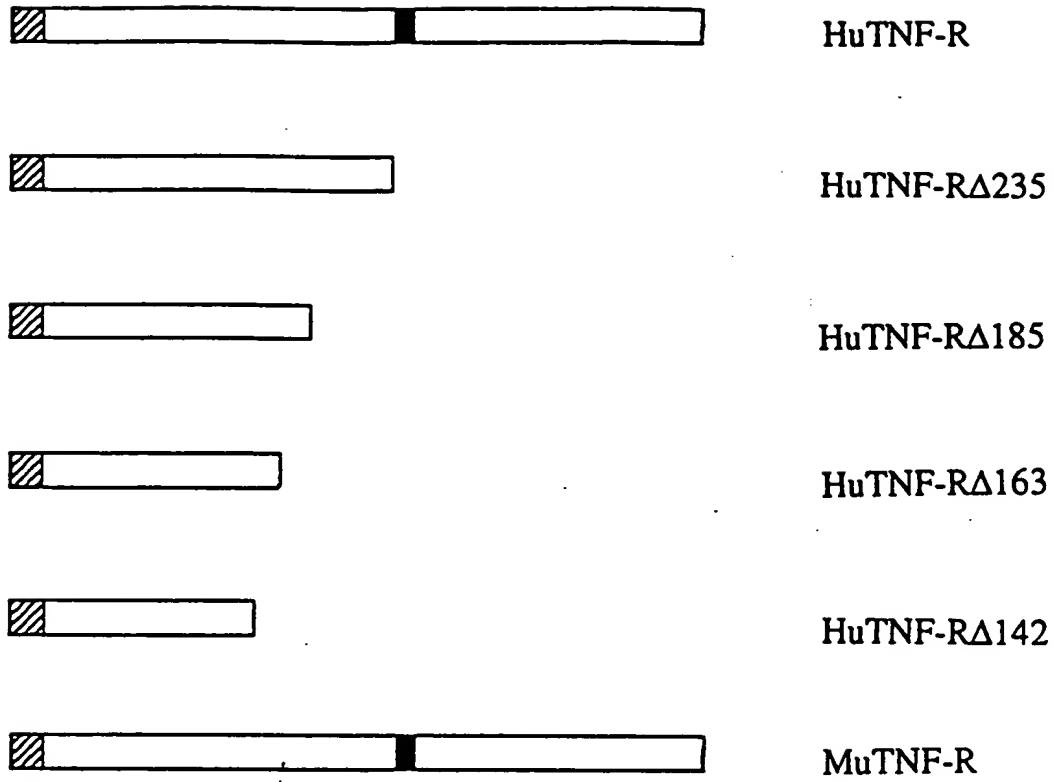


Figure 2A

GCGAGGCAGGCAGCCTGGAGAGAAGGCG	28
CTGGGCTGCGAGGSCGCGAGGGCGCGAGGGCAGGGGGCAACCGGACCCCGCCCCCATCC	87
ATG GCG CCC GTC GCC GTC TGG GCC GCG CTG GCC GTC GGA CTG GAG	132
Met Ala Pro Val Ala Val Trp Ala Ala Leu Ala Val Gly Leu Glu	-2
CTC TGG GCT GCG GCG CAC GCC TTG CCC GCC CAG GTG GCA TTT ACA	177
Leu Trp Ala Ala Ala His Ala Leu Pro Ala Gln Val Ala Phe Thr	8
CCC TAC GCC CCG GAG CCC GGG AGC ACA TGC CGG CTC AGA GAA TAC	222
Pro Tyr Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr	23
TAT GAC CAG ACA GCT CAG ATG TGC TGC AGC AAA TGC TCG CCG GGC	267
Tyr Asp Gln Thr Ala Gln Met Cys Cys Ser Lys Cys Ser Pro Gly	38
CAA CAT GCA AAA GTC TTC TGT ACC AAG ACC TCG GAC ACC GTG TGT	312
Gln His Ala Lys Val Phe Cys Thr Lys Thr Ser Asp Thr Val Cys	53
GAC TCC TGT GAG GAC AGC ACA TAC ACC CAG CTC TGG AAC TGG GTT	357
Asp Ser Cys Glu Asp Ser Thr Tyr Thr Gln Leu Trp Asn Trp Val	62
CCC GAG TGC TTG AGC TGT GGC TCC CGC TGT AGC TCT GAC CAG GTG	402
Pro Glu Cys Leu Ser Cys Gly Ser Arg Cys Ser Ser Asp Gln Val	83
GAA ACT CAA GCC TGC ACT CGG GAA CAG AAC CGC ATC TGC ACC TGC	447
Glu Thr Gln Ala Cys Thr Arg Glu Gln Asn Arg Ile Cys Thr Cys	92
AGG CCC GGC TGG TAC TGC GCG CTG AGC AAG CAG GAG GGG TGC CGG	492
Arg Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys Arg	113
CTG TGC GCG CCG CTG CGC AAG TGC CGC CCG GGC TTC GGC GTG GCC	537
Leu Cys Ala Pro Leu Arg Lys Cys Arg Pro Gly Phe Gly Val Ala	129
AGA CCA GGA ACT GAA ACA TCA GAC GTG GTG TGC AAG CCC TGT GCC	582
Arg Pro Gly Thr Glu Thr Ser Asp Val Val Cys Lys Pro Cys Ala	143
CCG GGG ACG TTC TCC AAC ACG ACT TCA TCC ACG GAT ATT TGC AGG	627
Pro Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr Asp Ile Cys Arg	152
CCC CAC CAG ATC TGT AAC GTG GTG GCC ATC CCT GGG AAT GCA AGC	672
Pro His Gln Ile Cys Asn Val Val Ala Ile Pro Gly Asn Ala Ser	173
ATG GAT GCA GTC TGC ACG TCC ACG TCC CCC ACC CGG AGT ATG GCC	717
Met Asp Ala Val Cys Thr Ser Thr Ser Pro Thr Arg Ser Met Ala	188
CCA GGG GCA GTA CAC TTA CCC CAG CCA GTG TCC ACA CGA TCC CAA	762
Pro Gly Ala Val His Leu Pro Gln Pro Val Ser Thr Arg Ser Gln	203
CAC ACG CAG CCA ACT CCA GAA CCC AGC ACT GCT CCA AGC ACC TCC	807
His Thr Gln Pro Thr Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser	218
TTC CTG CTC CCA ATG GGC CCC AGC CCC CCA GCT GAA GGG AGC ACT	852
Phe Leu Leu Pro Met Gly Pro Ser Pro Pro Ala Glu Gly Ser Thr	233
GGC GAC TTC GCT CTT CCA GTT GGA CTG ATT GTG GGT GTG ACA GCC	897
Gly Asp Phe Ala Leu Pro Val Gly Leu Ile Val Gly Val Thr Ala	248
TTG GGT CTA CTA ATA ATA GGA GTG GTG AAC TGT GTC ATC ATG ACC	942
Leu Gly Leu Leu Ile Ile Gly Val Val Asn Cys Val Ile Met Thr	263

Figure 2B

CAG GTG AAA AAG AAG CCC TTG TGC CTG CAG AGA GAA GCC AAG GTG	987
<u>Gln Val</u> Lys Lys Lys Pro Leu Cys Leu Gln Arg Glu Ala Lys Val	278
CCT CAC TTG CCT GCC GAT AAG GCC CGG GGT ACA CAG GGC CCC GAG	1032
Pro His Leu Pro Ala Asp Lys Ala Arg Gly Thr Gln Gly Pro Glu	293
CAG CAG CAC CTG CTG ATC ACA GCG CCG AGC TCC AGC AGC AGC TCC	1077
Gln Gln His Leu Leu Ile Thr Ala Pro Ser Ser Ser Ser Ser Ser	308
CTG GAG AGC TCG GCC AGT GCG TTG GAC AGA AGG GCG CCC ACT CGG	1122
Leu Glu Ser Ser Ala Ser Ala Leu Asp Arg Arg Ala Pro Thr Arg	323
AAC CAG CCA CAG GCA CCA GGC GTG GAG GCC AGT GGG GCC GGG GAG	1167
Asn Gln Pro Gln Ala Pro Gly Val Glu Ala Ser Gly Ala Gly Glu	338
GCC CGG GCC AGC ACC GGG AGC TCA GAT TCT TCC CCT GGT GGC CAT	1212
Ala Arg Ala Ser Thr Gly Ser Ser Asp Ser Ser Pro Gly Gly His	353
GGG ACC CAG GTC AAT GTC ACC TGC ATC GTG AAC GTC TGT AGC AGC	1257
Gly Thr Gln Val Asn Val Thr Cys Ile Val Asn Val Cys Ser Ser	368
TCT GAC CAC AGC TCA CAG TGC TCC TCC CAA GCC AGC TCC ACA ATG	1302
Ser Asp His Ser Ser Gln Cys Ser Ser Gln Ala Ser Ser Thr Met	383
GGA GAC ACA GAT TCC AGC CCC TCG GAG TCC CCG AAG GAC GAG CAG	1347
Gly Asp Thr Asp Ser Ser Pro Ser Glu Ser Pro Lys Asp Glu Gln	398
GTC CCC TTC TCC AAG GAG GAA TGT GCC TTT CGG TCA CAG CTG GAG	1392
Val Pro Phe Ser Lys Glu Glu Cys Ala Phe Arg Ser Gln Leu Glu	413
ACG CCA GAG ACC CTG CTG GGG AGC ACC GAA GAG AAG CCC CTG CCC	1437
Thr Pro Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys Pro Leu Pro	428
CTT GGA GTG CCT GAT GCT GGG ATG AAG CCC AGT	1470
Leu Gly Val Pro Asp Ala Gly Met Lys Pro Ser	439
TAACCAGGCCGGTGTGGGCTGTGTCGTAGCCAAGGTGGGCTGAGCCCTGGCAGGATGAC	
CCTGCGAAGGGGCCCTGGTCCTTCCAGGCCCCCACCCTAGGACTCTGAGGCTCTTTCT	
GGGCCAAGTTCTCTAGTGCCCTCCACAGCCGCAGCCTCCCTCTGACCTGCAG...	

Figure 3A

CGCAGCTGAGGCACTAGAGCTCC															23
AGGCACAAGGGCGGGAGCCACCGCTGCCCCCT															75
Met Ala Pro Ala Ala Leu Trp															-16
GTC	GCG	CTG	GTC	TTC	GAA	CTG	CAG	CTG	TGG	GCC	ACC	GGG	CAC	ACA	120
Val	Ala	Leu	Val	Phe	Glu	Leu	Gln	Leu	Trp	Ala	Thr	Gly	His	Thr	-1
GTG	CCC	GCC	CAG	GTT	GTC	TTG	ACA	CCC	TAC	AAA	CCG	GAA	CCT	GGG	165
Val	Pro	Ala	Gln	Val	Val	Leu	Thr	Pro	Tyr	Lys	Pro	Glu	Pro	Gly	15
TAC	GAG	TGC	CAG	ATC	TCA	CAG	GAA	TAC	TAT	GAC	AGG	AAG	GCT	CAG	210
Tyr	Glu	Cys	Gln	Ile	Ser	Gln	Glu	Tyr	Tyr	Asp	Arg	Lys	Ala	Gln	30
ATG	TGC	TGT	GCT	AAG	TGT	CCT	CCT	GGC	CAA	TAT	GTG	AAA	CAT	TTC	255
Met	Cys	Cys	Ala	Lys	Cys	Pro	Pro	Gly	Gln	Tyr	Val	Lys	His	Phe	45
TGC	AAC	AAG	ACC	TCG	GAC	ACC	GTG	TGT	GCG	GAC	TGT	GAG	GCA	AGC	300
Cys	Asn	Lys	Thr	Ser	Asp	Thr	Val	Cys	Ala	Asp	Cys	Glu	Ala	Ser	60
ATG	TAT	ACC	CAG	GTC	TGG	AAC	CAG	TTT	CGT	ACA	TGT	TTG	AGC	TGC	345
Met	Tyr	Thr	Gln	Val	Trp	Asn	Gln	Phe	Arg	Thr	Cys	Leu	Ser	Cys	75
AGT	TCT	TCC	TGT	ACC	ACT	GAC	CAG	GTG	GAG	ATC	CGC	GCC	TGC	ACT	390
Ser	Ser	Ser	Cys	Thr	Thr	Asp	Gln	Val	Glu	Ile	Arg	Ala	Cys	Thr	90
AAA	CAG	CAG	AAC	CGA	GTG	TGT	GCT	TGC	GAA	GCT	GGC	AGG	TAC	Tgc	435
Lys	Gln	Gln	Asn	Arg	Val	Cys	Ala	Cys	Glu	Ala	Gly	Arg	Tyr	Cys	105
GCC	TTG	AAA	ACC	CAT	TCT	GGC	AGC	TGT	CGA	CAG	TGC	ATG	AGG	CTG	480
Ala	Leu	Lys	Thr	His	Ser	Gly	Ser	Cys	Arg	Gln	Cys	Met	Arg	Leu	120
AGC	AAG	TGC	GGC	CCT	GGC	TTC	GGA	GTG	GCC	AGT	TCA	AGA	GCC	CCA	525
Ser	Lys	Cys	Gly	Pro	Gly	Phe	Gly	Val	Ala	Ser	Ser	Arg	Ala	Pro	135
AAT	GGA	AAT	GTG	CTA	TGC	AAG	GCC	TGT	GCC	CCA	GGG	ACG	TTC	TCT	570
Asn	Gly	Asn	Val	Leu	Cys	Lys	Ala	Cys	Ala	Pro	Gly	Thr	Phe	Ser	150
GAC	ACC	ACA	TCA	TCC	ACT	GAT	GTG	TGC	AGG	CCC	CAC	CGC	ATC	TGT	615
Asp	Thr	Thr	Ser	Ser	Thr	Asp	Val	Cys	Arg	Pro	His	Arg	Ile	Cys	165
AGC	ATC	CTG	GCT	ATT	CCC	GGA	AAT	GCA	AGC	ACA	GAT	GCA	GTC	TGT	660
Ser	Ile	Leu	Ala	Ile	Pro	Gly	Asn	Ala	Ser	Thr	Asp	Ala	Val	Cys	180
GCG	CCC	GAG	TCC	CCA	ACT	CTA	AGT	GCC	ATC	CCA	AGG	ACA	CTC	TAC	705
Ala	Pro	Glu	Ser	Pro	Thr	Leu	Ser	Ala	Ile	Pro	Arg	Thr	Leu	Tyr	195
GTA	TCT	CAG	CCA	GAG	CCC	ACA	AGA	TCC	CAA	CCC	CTG	GAT	CAA	GAG	750
Val	Ser	Gln	Pro	Glu	Pro	Thr	Arg	Ser	Gln	Pro	Leu	Asp	Gln	Glu	210
CCA	GGG	CCC	AGC	CAA	ACT	CCA	AGC	ATC	CTT	ACA	TCG	TTG	GGT	TCA	795
Pro	Gly	Pro	Ser	Gln	Thr	Pro	Ser	Ile	Leu	Thr	Ser	Leu	Gly	Ser	225
ACC	CCC	ATT	ATT	GAA	CAA	AGT	ACC	AAG	GGT	GGC	ATC	TCT	CTT	CCA	840
Thr	Pro	Ile	Ile	Glu	Gln	Ser	Thr	Lys	Gly	Gly	Ile	Ser	Leu	Pro	240
ATT	GGT	CTG	ATT	GTT	GGA	GTG	ACA	TCA	CTG	GGT	CTG	CTG	ATG	TTA	885
Ile	Gly	Leu	Ile	Val	Gly	Val	Thr	Ser	Leu	Gly	Leu	Leu	Met	Leu	255

Figure 3B

GGA CTG GTG AAC TGC ATC ATC CTG GTG CAG AGG AAA AAG AAG CCC	930
Gly Leu Val Asn Cys Ile Ile Leu Val Gln Arg Lys Lys Lys Pro	270
TCC TGC CTA CAA AGA GAT GCC AAG GTG CCT CAT GTG CCT GAT GAG	975
Ser Cys Leu Gln Arg Asp Ala Lys Val Pro His Val Pro Asp Glu	285
AAA TCC CAG GAT GCA GTA GGC CTT GAG CAG CAG CAC CTG TTG ACC	1020
Lys Ser Gln Asp Ala Val Gly Leu Glu Gln Gln His Leu Leu Thr	300
AL GCA CCC AGT TCC AGC AGC AGC TCC CTA GAG AGC TCA GCC AGC	1065
Thr Ala Pro Ser Ser Ser Ser Ser Ser Leu Glu Ser Ser Ala Ser	315
GCT GGG GAC CGA AGG GCG CCC CCT GGG GGC CAT CCC CAA GCA AGA	1110
Ala Gly Asp Arg Arg Ala Pro Pro Gly Gly His Pro Gln Ala Arg	330
GTC ATG GCG GAG GCC CAA GGG TTT CAG GAG GCC CGT GCC AGC TCC	1155
Val Met Ala Glu Ala Gln Gly Phe Gln Glu Ala Arg Ala Ser Ser	345
AGG ATT TCA GAT TCT TCC CAC GGA AGC CAC GGG ACC CAC GTC AAC	1200
Arg Ile Ser Asp Ser Ser His Gly Ser His Gly Thr His Val Asn	360
GTC ACC TGC ATC GTG AAC GTC TGT AGC AGC TCT GAC CAC AGT TCT	1245
Val Thr Cys Ile Val Asn Val Cys Ser Ser Ser Asp His Ser Ser	375
CAG TGC TCT TCC CAA GCC AGC GCC ACA GTG GGA GAC CCA GAT GCC	1290
Gln Cys Ser Ser Gln Ala Ser Ala Thr Val Gly Asp Pro Asp Ala	390
AAG CCC TCA GCG TCC CCA AAG GAT GAG CAG GTC CCC TTC TCT CAG	1335
Lys Pro Ser Ala Ser Pro Lys Asp Glu Gln Val Pro Phe Ser Gln	405
GAG GAG TGT CCG TCT CAG TCC CCG TGT GAG ACT ACA GAG ACA CTG	1380
Glu Glu Cys Pro Ser Gln Ser Pro Cys Glu Thr Thr Glu Thr Leu	420
CAG AGC CAT GAG AAG CCC TTG CCC CTT GGT GTG CCG GAT ATG GGC	1425
Gln Ser His Glu Lys Pro Leu Pro Leu Gly Val Pro Asp Met Gly	435
ATG AAG CCC AGC CAA GCT GGC TGG TTT GAT CAG ATT GCA GTC AAA	1470
Met Lys Pro Ser Gln Ala Gly Trp Phe Asp Gln Ile Ala Val Lys	450
GTG GCC	1476
Val Ala	452
TGACCCCTGACAGGGGTAACACCCTGCAAAGGGACCCCCGAGACCCTGAACCCATGGAAC	1536
TTTCATGACTTTTGCTGGATCCATTTCCCTTAGTGGCTTCCAGAGCCCCAGTTGCAGGTCA	1596
AGTGAGGGCTGAGACAGCTAGAGTGGTCAAAAAGTGCATGGTGTATTTATGGGGCAGTC	1656
CCAGGAAGTTGTTGCTCTTCCATGACCCCTCTGGATCTCTGGGCTCTTGCCTGATTCTT	1716
GCTTCTGAGAGGCCCCAGTATTTTTTCTTCTAAGGAGCTAACATCCTCTTCCATGAATA	1776
GCACAGCTCTTCAGCCTGAATGCTGACACTGCAGGGCGGTTCAGCAAGTAGGAGCAAGT	1836
GGTGGCCTGGTAGGGCACAGAGGCCCTTCAGGTTAGTGCTAAACTCTTAGGAAGTACCCT	1896
CTCCAAGCCCAACGAAATCTTTTGATGCAAGAATCAGAGGCCCATCAGGCAGAGTTGC	1956
TCTGTTATAGGATGGTAGGGCTGTAAGTCAgTGGTCCAgTGTGCTTTTAGCATGCCCTGG	2016
GTTTGATCCTCAGCAACACATGCAAAACGTAAGTAGACAGCAGACAGCAGACAGC	2076
CAGCCCCCTGTGTGGTTTGAGCCTCTGCCTTTGACTTTACTCTGGTGGGCACACAGAG	2136
GGCTGGAGCTCCTCCTGACCTTCTAATGAGCCCTTCCAAGGCCACGCCTTCCTTCAG	2196
GGAACTCTCAGGGACTGTAGAGTTCCCAGGCCCTGCAGCCACCTGTCTCTTCTACCTCA	2256
GCCTGGAGCACTCCCTCTAAGTCCCAACGCTTGGTACTGTACTTGTGTGACCCCAAC	2316
GTGCATTGTCCGGGTTAGGCACTGTGAGTTGGAACAGCTCATGACATCGGTTGAAAGGCC	2376
CACCCGGAACAGCTAAGCCAGCTCTTTTGCCAAAGGATTGATGCCGGTTTCTAATCAa	2436
CCTGCTCCCTAGCATTGCCTGGAAGGAAAGGGTTTCAAGAGACTCCTCAAGAAGCAAGTTC	2496
AGTCTCAGGTGCTTGGATGCCATGCTCACCGATTCCACTGGATATGAACTGGCAGAGGA	2556

Figure 3C

GCCTAGTTGTTGCCATGGAGACTTAAAGAGCTCAGCACTCTGGAATCAAGATACTGGACA	2616
CTTGGGGCCGACTTGTTAAGGCTCTGCAGCATCAGACTGTAGAGGGGAAGGAACACGTCT	2676
GCCCCCTGGTGGCCCGTCCTGGGAAGACCTCGGGCCCTCCTAGGCAACAAAAGAATGAATT	2736
GGAAAGGATGTTCTGGGTGTGGCCTAGCTCCTGTGCTTGTGTGGATCCCTAAAGGGTGT	2796
GCTAAGGAGCAATTGCACTGTGTGCTGGACAGAATTCCTGCTTATAAATGCTTTTTGTTG	2856
TTGTTTTGTACACTGAGCCCTGGCTGAGCCACCCACCCACCTCCCATCCCACCTTTAC	2916
ACGCCACTCTTGCAATGAGAACCTGGCTGTCTCCCACTTGTAGCCTGTGGATGCTGAGGAA	2976
ACACCCAGCCAAGTAGACTCCAGGCTTgCCCCTATCTCCTGcTaTGAGTcTg9CCTCCTC	3036
AtTgTGTGTGGGAAGGAGACGGGtTCTGTcATCTCGGAAcgCCCACACCGTGGATGTGA	3096
ACAaTGGCTGTACTAGCTTAGACCagCTTAGGGCTCTGCATATCACAGGAGGGGGAGCAG	3156
GGAACAATTTGAGTGCTGACCTATAACACAgTTCCTAAAGGATCGGGCAGTCCAGAATCT	3216
CCTCCTTCAGT	3276
TGCATGTATGTGTGTGCCAGTGTGTGGAGGCCCGAGGTTGGCTTTGGGTGTGTTGATCA	3336
CTCTCCAGTTACTGAGCGGGCTCTCATCTGTACCCAGAGCTTGACATTTTCTAGTCTA	3396
ACTTGATTcAGGGATCTCTGTCTGCCTATGGAGgTGCTCAGGTTACAGGCAGGCTGCCAT	3456
ACCTGCCCCGACATTTACATGAATACTAGAGATCTGAATTCTGGTCCTCACACTTGTATAC	3516
CTGCATTTTATCCACTAAGACATCTCTCCAAGGGCTCCCCCTTCCTATTTAATAAGTTAG	3576
TTTTGAActGGCAAGATGGCTCAGTGGGTAAGGCAGTTTGCGGACAAACCTGATGACCTG	3636
AGTTGGATCCCTGACCATAAGGTAGAAGAGACCTGATTCCCTGCAAGTTGTCTCTGACCA	3696
CCACCCCATACATGCTTCTGCATATGTGCACACATCACATTCTTGACACACACTCACAT	3756
ACCATAAATGTAATAAATTTTTTTAAATAAATTGATTTTATCTTTTAAAAAAAAAAAAA	3813

FILING RECEIPT



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APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTORNEY DOCKET NO.	DRWGS	TOT CL	IND CL
09/144,502	08/31/98	1646	\$3,084.00	A-7210	6	87	17

SUGHRUE MION ZINN MACPEAK & SEAS
2100 PENNSYLVANIA AVENUE NW
WASHINGTON DC 20037-3202

Receipt is acknowledged of this nonprovisional Patent Application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please write to the Application Processing Division's Customer Correction Branch within 10 days of receipt. Please provide a copy of the Filing Receipt with the changes noted thereon.

Applicant(s)

CRAIG A. SMITH, SEATTLE, WA; RAYMOND G. GOODWIN,
SEATTLE, WA; M. PATRICIA BECKMANN, POULSBO, WA.

CONTINUING DATA AS CLAIMED BY APPLICANT-

THIS APPLN IS A RE OF	08/346,555	11/29/94	PAT 5,712,155
WHICH IS A CON OF	07/523,635	05/10/90	PAT 5,395,760
WHICH IS A CIP OF	07/421,417	10/13/89	ABN
WHICH IS A CIP OF	07/405,370	09/11/89	ABN
WHICH IS A CIP OF	07/403,241	09/05/89	ABN

FOREIGN FILING LICENSE GRANTED 09/24/98

TITLE

DNA ENCODING TUMOR NECROSIS FACTOR-ALPHA AND -BETA RECEPTORS

PRELIMINARY CLASS: 435

1

PLEASE DATE STAMP AND RETURN TO US - BOX 235X

In re Application of:

CRAIG A. SMITH et al

Appln. No.: 09/144,502

Group Art Unit: 1646

Filed: August 31, 1998

Examiner: Fitzgerald, D.

For: **DNA ENCODING TUMOR NECROSIS
FACTOR- α AND - β RECEPTORS**

- PAPERS ENTITLED:
- (1) NOTICE TO COMPLY WITH REQUIREMENTS
FOR PATENT APPLICATIONS CONTAINING
NUCLEOTIDE SEQUENCE AND/OR AMINO
ACID SEQUENCE DISCLOSURES;
 - (2) PRELIMINARY AMENDMENT; and
 - (3) STATEMENT IN SUPPORT OF SUBMISSION
(accompanied by Sequence Listing
and DOS Version diskette containing
same).

**SUGHRUE, MION, ZINN,
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(202) 293-7060

DOCKET NO.: 98-7210
ATTORNEY: GR/vf

Date: October 9, 1998

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

CRAIG A. SMITH et al

Appln. No.: 09/144,502

Group Art Unit: 1646

Filed: August 31, 1998

Examiner: Fitzgerald, D.

For: DNA ENCODING TUMOR NECROSIS
FACTOR- α AND - β RECEPTORS

PRELIMINARY AMENDMENT

FILED
OCT 09 1998

Assistant Commissioner
for Patents
Washington, D.C. 20231

Sir:

Prior to examining the above-identified reissue application,
please amend the application as follows.

IN THE SPECIFICATION:

Please amend the specification as follows:

Page 4, lines 17-28, delete in their entirety.

Page 5, line 25, change "Figure 2A" to -- SEQ ID NO:1 --.

Page 6, line 6, change "Figure 2A" to -- SEQ ID NO:1 --; and
line 11, change "Figure 2A" to -- SEQ ID NO:1 --.

Page 8, lines 27-28, change "Figures 2-3 or Figures 4-6" to
-- SEQ ID NO:1 or SEQ ID NO:3 --.

Page 9, line 23, change "clone 11" to -- clone 1 --;
line 25, change "clone 11" to -- clone 1 --; and
line 26, change "Figures 4-6" to -- SEQ ID NO:1 --.

Page 13, line 21, change "Figure 2A" to -- SEQ ID NO:1 --;
line 23, change "transmembrane" to
-- extracellular --;

PRELIMINARY AMENDMENT
U.S. Appln. No. 09/144,502

line 25, change "Figure 2A" to -- SEQ ID NO:1 --; and

line 26, change "Figure 2A" to -- SEQ ID NO:1 --.

Page 15, line 13, change "Figure 2A" to -- SEQ ID NO:1 --.

Page 30, line 24, change "Figure 2A" to -- SEQ ID NO:1 --; and

line 28, after "oligonucleotides", insert
-- (encoding amino acids corresponding to Ala²²⁹-Asp²³⁵ of SEQ ID
NO:1) --.

Page 31, line 18, change "Figure 2A" to -- SEQ ID NO:1 --; and

line 22, after "linkers", insert -- (encoding amino
acids corresponding to Ile¹⁶²-Ala¹⁷⁶ and Val¹⁷⁷-Arg¹⁸⁵ of SEQ ID
NO:1) --.

Page 32, line 10, change "Figure 2A" to -- SEQ ID NO:1 --;

line 12, after "linkers", insert -- (encoding amino
acids corresponding to Ile¹⁶²-Cys¹⁶³ of SEQ ID NO:1) --; and

line 32, change "Figure 2A" to -- SEQ ID NO:1 --.

Page 33, line 2, after "linker", insert -- (encoding amino
acids corresponding to Thr¹³²-Cys¹⁴² of SEQ ID NO:1) --.

Page 36, line 2, after "primers", insert -- (encoding amino
acids corresponding in part to amino acids Leu¹-Thr⁸ and Pro²²⁵-Asp²³⁵
of SEQ ID NO:1) --.

Page 38, line 29, change "Figures 3A-3B" to -- SEQ ID NO:3 and
SEQ ID NO:4 --.

Page 40, after line 2, insert

-- DETAILED DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO:1 and SEQ ID NO:2 show the partial cDNA sequence and derived amino acid sequence of the human TNF-R clone 1. Nucleotides are numbered from the beginning of the 5' untranslated region. Amino acids are numbered from the beginning of the signal peptide sequence. The putative signal sequence is represented by amino acid -22 to -1. The N-terminus of the mature TNF-R begins with amino acid 1. The predicted transmembrane region extends from amino acids 236-265.

SEQ ID NO:3 and SEQ ID NO:4 show the cDNA sequence and derived amino acid sequence of murine TNF-R clone 11. The putative signal peptide sequence is represented by amino acids -22 to -1. The N-terminus of the mature TNF-R protein begins with amino acid 1. The predicted transmembrane region extends from amino acids 234 to 265. --

Pages 41-53, please renumber as new pages 54-66, respectively.

IN THE SEQUENCE LISTING:

Please insert the Sequence Listing (i.e., new pages 41-53) being filed simultaneously herewith.

IN THE DRAWINGS:

Please delete Figures 2A-2B and 3A-3C (in their entirety).

PRELIMINARY AMENDMENT
U.S. Appln. No. 09/144,502

REMARKS

The amendments to the specification were made in order for the present application to be consistent with Parent Application Serial No. 07/523,635, filed November 29, 1994 (now U.S. Patent No. 5,395,760)^{1/}; and to correct obvious typographical errors therein.

The Sequence Listing, filed simultaneously herewith, is being submitted and the specification is also being amended to be consistent with the amendments made in related Application Serial No. 08/650,000, filed June 9, 1998 (now allowed).

Also, the deletion of Figures 2-3 (in their entirety) and insertion of SEQ ID NOs:1-4 therein is made in order for the Sequence Listing and drawings to be consistent with related Application Serial No. 08/650,000, filed June 9, 1998 (now allowed).

Hence, the amendments to the specification, the insertion of the Sequence Listing and deletion of Figures 2-3 do not constitute new matter, and thus entry is requested.

^{1/} Note, due to a printing error, column 6, line 55, of U.S. Patent 5,395,760 refers to "Figures 2A-2C", rather than "Figures 2A-2B".

PRELIMINARY AMENDMENT
U.S. Appln. No. 09/144,502

The Examiner is invited to contact the undersigned at his Washington telephone number on any questions which might arise.

Respectfully submitted,



Gordon Kit
Registration No. 30,764

SUGHRUE, MION, ZINN,
MACPEAK & SEAS, PLLC
2100 Pennsylvania Avenue, N.W.
Washington, D.C. 20037-3202
(202) 293-7060

Date: October 9, 1998

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

CRAIG A. SMITH et al

Appln. No.: 09/144,502

Group Art Unit: 1646

Filed: August 31, 1998

Examiner: Fitzgerald, D.

For: DNA ENCODING TUMOR NECROSIS
FACTOR- α AND - β RECEPTORS

FILED
OCT 09 1998

STATEMENT IN SUPPORT OF SUBMISSION IN
ACCORDANCE WITH FORMER 37 C.F.R. §§ 1.821-1.825

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

The following statement is provided to meet the requirements of Former 37 C.F.R. §§ 1.821-1.825.

I hereby state that the content of the computer readable copy of the Sequence Listing submitted in accordance with Former 37 C.F.R. §§ 1.821-1.825, respectively, is the same as the Sequence Listing filed simultaneously herewith.

The Examiner is requested to note that the Sequence Listing being filed herewith is submitted under Former 37 C.F.R. §§ 1.821-1.825, as the present application is a reissue application of U.S. Patent No. 5,712,155 which issued January 27, 1998, based on U.S. Patent Application No. 08/346,555, filed November 29, 1994, i.e., prior to the effective new rules dated of July 1, 1998 (see 63 Federal Register 29620).

STATEMENT IN SUPPORT OF SUBMISSION IN
ACCORDANCE WITH FORMER 37 C.F.R. §§ 1.821-1.825
U.S. Appln. No. 09/144,502

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge and that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

10/9/98

Date



Gordon Kit

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: SMITH, Craig A.
GOODWIN, Raymond G.
BECKMANN, M. Patricia
- (ii) TITLE OF INVENTION: DNA ENCODING TUMOR NECROSIS
FACTOR- α AND - β RECEPTORS
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: IMMUNEX CORPORATION
 - (B) STREET: 51 University Street
 - (C) CITY: Seattle
 - (D) STATE: WASHINGTON
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 98101
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: 09/144,502
 - (B) FILING DATE: 31-AUG-1998
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/346,555
 - (B) FILING DATE: 29-NOV-1994
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 07/523,635
 - (B) FILING DATE: 10-MAY-1990
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 07/421,417
 - (B) FILING DATE: 13-OCT-1989
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 07/405,370
 - (B) FILING DATE: 11-SEPT-1989
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 07/403,241
 - (B) FILING DATE: 05-SEPT-1989

(viii) ATTORNEY/AGENT INFORMATION:
 (A) NAME: KIT, Gordon
 (B) REGISTRATION NUMBER: 30,764
 (C) REFERENCE/DOCKET NUMBER: A-7210

(ix) TELECOMMUNICATION INFORMATION:
 (A) TELEPHONE: (202) 293-7060
 (B) TELEFAX: (202) 293-7860

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1641 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens
 (G) CELL TYPE: Fibroblast
 (H) CELL LINE: WI-26 VA4

(vii) IMMEDIATE SOURCE:
 (A) LIBRARY: WI-26 VA4
 (B) CLONE: 1

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 88..1473

(ix) FEATURE:
 (A) NAME/KEY: mat_peptide
 (B) LOCATION: 154..1470

(ix) FEATURE:
 (A) NAME/KEY: sig_peptide
 (B) LOCATION: 88..153

(x) PUBLICATION INFORMATION:
 (A) AUTHORS: Smith, Craig A.
 Davis, Terri
 Anderson, Dirk
 Solam, Lisabeth
 Beckmann, M. P.
 Jerzy, Rita
 Dower, Steven K.
 Cosman, David
 Goodwin, Raymond G.

(B) TITLE: A Receptor for Tumor Necrosis
Factor Defines an Unusual Family
of Cellular and Viral Proteins

(C) JOURNAL: Science

(D) VOLUME: 248

(F) PAGES: 1019-1023

(G) DATE: 25-MAY-1990

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGAGGCAGG CAGCCTGGAG AGAAGGCGCT GGGCTGCGAG GGCGCGAGGG CGCGAGGGCA	60
GGGGGCAACC GGACCCCGCC CGCATCC ATG GCG CCC GTC GCC GTC TGG GCC	111
Met Ala Pro Val Ala Val Trp Ala	
-22 -20 -15	
GCG CTG GCC GTC GGA CTG GAG CTC TGG GCT GCG GCG CAC GCC TTG CCC	159
Ala Leu Ala Val Gly Leu Glu Leu Trp Ala Ala Ala His Ala Leu Pro	
-10 -5 1	
GCC CAG GTG GCA TTT ACA CCC TAC GCC CCG GAG CCC GGG AGC ACA TGC	207
Ala Gln Val Ala Phe Thr Pro Tyr Ala Pro Glu Pro Gly Ser Thr Cys	
5 10 15	
CGG CTC AGA GAA TAC TAT GAC CAG ACA GCT CAG ATG TGC TGC AGC AAA	255
Arg Leu Arg Glu Tyr Tyr Asp Gln Thr Ala Gln Met Cys Cys Ser Lys	
20 25 30	
TGC TCG CCG GGC CAA CAT GCA AAA GTC TTC TGT ACC AAG ACC TCG GAC	303
Cys Ser Pro Gly Gln His Ala Lys Val Phe Cys Thr Lys Thr Ser Asp	
35 40 45 50	
ACC GTG TGT GAC TCC TGT GAG GAC AGC ACA TAC ACC CAG CTC TGG AAC	351
Thr Val Cys Asp Ser Cys Glu Asp Ser Thr Tyr Thr Gln Leu Trp Asn	
55 60 65	
TGG GTT CCC GAG TGC TTG AGC TGT GGC TCC CGC TGT AGC TCT GAC CAG	399
Trp Val Pro Glu Cys Leu Ser Cys Gly Ser Arg Cys Ser Ser Asp Gln	
70 75 80	
GTG GAA ACT CAA GCC TGC ACT CGG GAA CAG AAC CGC ATC TGC ACC TGC	447
Val Glu Thr Gln Ala Cys Thr Arg Glu Gln Asn Arg Ile Cys Thr Cys	
85 90 95	
AGG CCC GGC TGG TAC TGC GCG CTG AGC AAG CAG GAG GGG TGC CGG CTG	495
Arg Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys Arg Leu	
100 105 110	
TGC GCG CCG CTG CGC AAG TGC CGC CCG GGC TTC GGC GTG GCC AGA CCA	543
Cys Ala Pro Leu Arg Lys Cys Arg Pro Gly Phe Gly Val Ala Arg Pro	
115 120 125 130	
GGA ACT GAA ACA TCA GAC GTG GTG TGC AAG CCC TGT GCC CCG GGG ACG	591
Gly Thr Glu Thr Ser Asp Val Val Cys Lys Pro Cys Ala Pro Gly Thr	
135 140 145	

TTC	TCC	AAC	ACG	ACT	TCA	TCC	ACG	GAT	ATT	TGC	AGG	CCC	CAC	CAG	ATC	639
Phe	Ser	Asn	Thr	Thr	Ser	Ser	Thr	Asp	Ile	Cys	Arg	Pro	His	Gln	Ile	
			150					155					160			
TGT	AAC	GTG	GTG	GCC	ATC	CCT	GGG	AAT	GCA	AGC	ATG	GAT	GCA	GTC	TGC	687
Cys	Asn	Val	Val	Ala	Ile	Pro	Gly	Asn	Ala	Ser	Met	Asp	Ala	Val	Cys	
		165					170					175				
ACG	TCC	ACG	TCC	CCC	ACC	CGG	AGT	ATG	GCC	CCA	GGG	GCA	GTA	CAC	TTA	735
Thr	Ser	Thr	Ser	Pro	Thr	Arg	Ser	Met	Ala	Pro	Gly	Ala	Val	His	Leu	
	180					185					190					
CCC	CAG	CCA	GTG	TCC	ACA	CGA	TCC	CAA	CAC	ACG	CAG	CCA	ACT	CCA	GAA	783
Pro	Gln	Pro	Val	Ser	Thr	Arg	Ser	Gln	His	Thr	Gln	Pro	Thr	Pro	Glu	
195					200					205					210	
CCC	AGC	ACT	GCT	CCA	AGC	ACC	TCC	TTC	CTG	CTC	CCA	ATG	GGC	CCC	AGC	831
Pro	Ser	Thr	Ala	Pro	Ser	Thr	Ser	Phe	Leu	Leu	Pro	Met	Gly	Pro	Ser	
				215					220					225		
CCC	CCA	GCT	GAA	GGG	AGC	ACT	GGC	GAC	TTC	GCT	CTT	CCA	GTT	GGA	CTG	879
Pro	Pro	Ala	Glu	Gly	Ser	Thr	Gly	Asp	Phe	Ala	Leu	Pro	Val	Gly	Leu	
			230					235					240			
ATT	GTG	GGT	GTG	ACA	GCC	TTG	GGT	CTA	CTA	ATA	ATA	GGA	GTG	GTG	AAC	927
Ile	Val	Gly	Val	Thr	Ala	Leu	Gly	Leu	Leu	Ile	Ile	Gly	Val	Val	Asn	
		245					250					255				
TGT	GTC	ATC	ATG	ACC	CAG	GTG	AAA	AAG	AAG	CCC	TTG	TGC	CTG	CAG	AGA	975
Cys	Val	Ile	Met	Thr	Gln	Val	Lys	Lys	Lys	Pro	Leu	Cys	Leu	Gln	Arg	
	260					265					270					
GAA	GCC	AAG	GTG	CCT	CAC	TTG	CCT	GCC	GAT	AAG	GCC	CGG	GGT	ACA	CAG	1023
Glu	Ala	Lys	Val	Pro	His	Leu	Pro	Ala	Asp	Lys	Ala	Arg	Gly	Thr	Gln	
275					280					285					290	
GGC	CCC	GAG	CAG	CAG	CAC	CTG	CTG	ATC	ACA	GCG	CCG	AGC	TCC	AGC	AGC	1071
Gly	Pro	Glu	Gln	Gln	His	Leu	Leu	Ile	Thr	Ala	Pro	Ser	Ser	Ser	Ser	
				295					300					305		
AGC	TCC	CTG	GAG	AGC	TCG	GCC	AGT	GCG	TTG	GAC	AGA	AGG	GCG	CCC	ACT	1119
Ser	Ser	Leu	Glu	Ser	Ser	Ala	Ser	Ala	Leu	Asp	Arg	Arg	Ala	Pro	Thr	
			310					315					320			
CGG	AAC	CAG	CCA	CAG	GCA	CCA	GGC	GTG	GAG	GCC	AGT	GGG	GCC	GGG	GAG	1167
Arg	Asn	Gln	Pro	Gln	Ala	Pro	Gly	Val	Glu	Ala	Ser	Gly	Ala	Gly	Glu	
		325					330					335				
GCC	CGG	GCC	AGC	ACC	GGG	AGC	TCA	GAT	TCT	TCC	CCT	GGT	GGC	CAT	GGG	1215
Ala	Arg	Ala	Ser	Thr	Gly	Ser	Ser	Asp	Ser	Ser	Pro	Gly	Gly	His	Gly	
	340					345					350					
ACC	CAG	GTC	AAT	GTC	ACC	TGC	ATC	GTG	AAC	GTC	TGT	AGC	AGC	TCT	GAC	1263
Thr	Gln	Val	Asn	Val	Thr	Cys	Ile	Val	Asn	Val	Cys	Ser	Ser	Ser	Asp	
355					360					365					370	

CAC AGC TCA CAG TGC TCC TCC CAA GCC AGC TCC ACA ATG GGA GAC ACA	1311
His Ser Ser Gln Cys Ser Ser Gln Ala Ser Ser Thr Met Gly Asp Thr	
375 380 385	
GAT TCC AGC CCC TCG GAG TCC CCG AAG GAC GAG CAG GTC CCC TTC TCC	1359
Asp Ser Ser Pro Ser Glu Ser Pro Lys Asp Glu Gln Val Pro Phe Ser	
390 395 400	
AAG GAG GAA TGT GCC TTT CGG TCA CAG CTG GAG ACG CCA GAG ACC CTG	1407
Lys Glu Glu Cys Ala Phe Arg Ser Gln Leu Glu Thr Pro Glu Thr Leu	
405 410 415	
CTG GGG AGC ACC GAA GAG AAG CCC CTG CCC CTT GGA GTG CCT GAT GCT	1455
Leu Gly Ser Thr Glu Glu Lys Pro Leu Pro Leu Gly Val Pro Asp Ala	
420 425 430	
GGG ATG AAG CCC AGT TAACCAGGCC GGTGTGGGCT GTGTCGTAGC CAAGGTGGGC	1510
Gly Met Lys Pro Ser	
435 440	
TGAGCCCTGG CAGGATGACC CTGCGAAGGG GCCCTGGTCC TTCCAGGCCC CCACCACTAG	1570
GACTCTGAGG CTCTTTCTGG GCCAAGTTCC TCTAGTGCCC TCCACAGCCG CAGCCTCCCT	1630
CTGACCTGCA G	1641

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 461 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Pro Val Ala Val Trp Ala Ala Leu Ala Val Gly Leu Glu Leu	
-22 -20 -15 -10	
Trp Ala Ala Ala His Ala Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr	
-5 1 5 10	
Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln	
15 20 25	
Thr Ala Gln Met Cys Cys Ser Lys Cys Ser Pro Gly Gln His Ala Lys	
30 35 40	
Val Phe Cys Thr Lys Thr Ser Asp Thr Val Cys Asp Ser Cys Glu Asp	
45 50 55	
Ser Thr Tyr Thr Gln Leu Trp Asn Trp Val Pro Glu Cys Leu Ser Cys	
60 65 70	

Gly	Ser	Arg	Cys	Ser	Ser	Asp	Gln	Val	Glu	Thr	Gln	Ala	Cys	Thr	Arg	75	80	85	90
Glu	Gln	Asn	Arg	Ile	Cys	Thr	Cys	Arg	Pro	Gly	Trp	Tyr	Cys	Ala	Leu	95	100	105	
Ser	Lys	Gln	Glu	Gly	Cys	Arg	Leu	Cys	Ala	Pro	Leu	Arg	Lys	Cys	Arg	110	115	120	
Pro	Gly	Phe	Gly	Val	Ala	Arg	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	125	130	135	
Cys	Lys	Pro	Cys	Ala	Pro	Gly	Thr	Phe	Ser	Asn	Thr	Thr	Ser	Ser	Thr	140	145	150	
Asp	Ile	Cys	Arg	Pro	His	Gln	Ile	Cys	Asn	Val	Val	Ala	Ile	Pro	Gly	155	160	165	170
Asn	Ala	Ser	Met	Asp	Ala	Val	Cys	Thr	Ser	Thr	Ser	Pro	Thr	Arg	Ser	175	180	185	
Met	Ala	Pro	Gly	Ala	Val	His	Leu	Pro	Gln	Pro	Val	Ser	Thr	Arg	Ser	190	195	200	
Gln	His	Thr	Gln	Pro	Thr	Pro	Glu	Pro	Ser	Thr	Ala	Pro	Ser	Thr	Ser	205	210	215	
Phe	Leu	Leu	Pro	Met	Gly	Pro	Ser	Pro	Pro	Ala	Glu	Gly	Ser	Thr	Gly	220	225	230	
Asp	Phe	Ala	Leu	Pro	Val	Gly	Leu	Ile	Val	Gly	Val	Thr	Ala	Leu	Gly	235	240	245	250
Leu	Leu	Ile	Ile	Gly	Val	Val	Asn	Cys	Val	Ile	Met	Thr	Gln	Val	Lys	255	260	265	
Lys	Lys	Pro	Leu	Cys	Leu	Gln	Arg	Glu	Ala	Lys	Val	Pro	His	Leu	Pro	270	275	280	
Ala	Asp	Lys	Ala	Arg	Gly	Thr	Gln	Gly	Pro	Glu	Gln	Gln	His	Leu	Leu	285	290	295	
Ile	Thr	Ala	Pro	Ser	Ser	Ser	Ser	Ser	Ser	Leu	Glu	Ser	Ser	Ala	Ser	300	305	310	
Ala	Leu	Asp	Arg	Arg	Ala	Pro	Thr	Arg	Asn	Gln	Pro	Gln	Ala	Pro	Gly	315	320	325	330
Val	Glu	Ala	Ser	Gly	Ala	Gly	Glu	Ala	Arg	Ala	Ser	Thr	Gly	Ser	Ser	335	340	345	
Asp	Ser	Ser	Pro	Gly	Gly	His	Gly	Thr	Gln	Val	Asn	Val	Thr	Cys	Ile	350	355	360	

Val	Asn	Val	Cys	Ser	Ser	Ser	Asp	His	Ser	Ser	Gln	Cys	Ser	Ser	Gln
		365					370					375			
Ala	Ser	Ser	Thr	Met	Gly	Asp	Thr	Asp	Ser	Ser	Pro	Ser	Glu	Ser	Pro
	380					385					390				
Lys	Asp	Glu	Gln	Val	Pro	Phe	Ser	Lys	Glu	Glu	Cys	Ala	Phe	Arg	Ser
395					400					405					410
Gln	Leu	Glu	Thr	Pro	Glu	Thr	Leu	Leu	Gly	Ser	Thr	Glu	Glu	Lys	Pro
				415					420					425	
Leu	Pro	Leu	Gly	Val	Pro	Asp	Ala	Gly	Met	Lys	Pro	Ser			
			430					435							

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3813 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA to mRNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: mouse
 - (B) STRAIN: C57BL/6
 - (G) CELL TYPE: T-helper cell
 - (H) CELL LINE: 7B9
- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: 11
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 55..1479
- (ix) FEATURE:
 - (A) NAME/KEY: mat_peptide
 - (B) LOCATION: 121..1476
- (ix) FEATURE:
 - (A) NAME/KEY: sig_peptide
 - (B) LOCATION: 55..120

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGCAGCTGAG GCACTAGAGC TCCAGGCACA AGGGCGGGAG CCACCGCTGC CCCT															ATG Met -22	57
GCG Ala	CCC Pro -20	GCC Ala	GCC Ala	CTC Leu	TGG Trp	GTC Val -15	GCG Ala	CTG Leu	GTC Val	TTC Phe	GAA Glu -10	CTG Leu	CAG Gln	CTG Leu	TGG Trp	105
GCC Ala -5	ACC Thr	GGG Gly	CAC His	ACA Thr	GTG Val 1	CCC Pro	GCC Ala	CAG Gln	GTT Val 5	GTC Val	TTG Leu	ACA Thr	CCC Pro	TAC Tyr 10	AAA Lys	153
CCG Pro	GAA Glu	CCT Pro	GGG Gly 15	TAC Tyr	GAG Glu	TGC Cys	CAG Gln	ATC Ile 20	TCA Ser	CAG Gln	GAA Glu	TAC Tyr	TAT Tyr 25	GAC Asp	AGG Arg	201
AAG Lys	GCT Ala	CAG Gln 30	ATG Met	TGC Cys	TGT Cys	GCT Ala	AAG Lys 35	TGT Cys	CCT Pro	CCT Pro	GGC Gly	CAA Gln 40	TAT Tyr	GTG Val	AAA Lys	249
CAT His	TTC Phe 45	TGC Cys	AAC Asn	AAG Lys	ACC Thr	TCG Ser 50	GAC Asp	ACC Thr	GTG Val	TGT Cys	GCG Ala 55	GAC Asp	TGT Cys	GAG Glu	GCA Ala	297
AGC Ser 60	ATG Met	TAT Tyr	ACC Thr	CAG Gln	GTC Val 65	TGG Trp	AAC Asn	CAG Gln	TTT Phe	CGT Arg 70	ACA Thr	TGT Cys	TTG Leu	AGC Ser	TGC Cys 75	345
AGT Ser	TCT Ser	TCC Ser	TGT Cys	ACC Thr 80	ACT Thr	GAC Asp	CAG Gln	GTG Val	GAG Glu 85	ATC Ile	CGC Arg	GCC Ala	TGC Cys	ACT Thr 90	AAA Lys	393
CAG Gln	CAG Gln	AAC Asn 95	CGA Arg	GTG Val	TGT Cys	GCT Ala	TGC Cys	GAA Glu 100	GCT Ala	GGC Gly	AGG Arg	TAC Tyr	TGC Cys 105	GCC Ala	TTG Leu	441
AAA Lys	ACC Thr	CAT His 110	TCT Ser	GGC Gly	AGC Ser	TGT Cys	CGA Arg 115	CAG Gln	TGC Cys	ATG Met	AGG Arg	CTG Leu 120	AGC Ser	AAG Lys	TGC Cys	489
GGC Gly	CCT Pro 125	GGC Gly	TTC Phe	GGA Gly	GTG Val	GCC Ala 130	AGT Ser	TCA Ser	AGA Arg	GCC Ala	CCA Pro 135	AAT Asn	GGA Gly	AAT Asn	GTG Val	537
CTA Leu 140	TGC Cys	AAG Lys	GCC Ala	TGT Cys	GCC Ala 145	CCA Pro	GGG Gly	ACG Thr	TTC Phe	TCT Ser 150	GAC Asp	ACC Thr	ACA Thr	TCA Ser	TCC Ser 155	585
ACT Thr	GAT Asp	GTG Val	TGC Cys	AGG Arg 160	CCC Pro	CAC His	CGC Arg	ATC Ile	TGT Cys 165	AGC Ser	ATC Ile	CTG Leu	GCT Ala	ATT Ile 170	CCC Pro	633

GGA Gly	AAT Asn	GCA Ala	AGC Ser 175	ACA Thr	GAT Asp	GCA Ala	GTC Val 180	TGT Cys 180	GCG Ala	CCC Pro	GAG Glu	TCC Ser 185	CCA Pro 185	ACT Thr	CTA Leu	681
AGT Ser	GCC Ala	ATC Ile 190	CCA Pro	AGG Arg	ACA Thr	CTC Leu	TAC Tyr 195	GTA Val	TCT Ser	CAG Gln	CCA Pro	GAG Glu 200	CCC Pro	ACA Thr	AGA Arg	729
TCC Ser	CAA Gln 205	CCC Pro	CTG Leu	GAT Asp	CAA Gln	GAG Glu 210	CCA Pro	GGG Gly	CCC Pro	AGC Ser	CAA Gln 215	ACT Thr	CCA Pro	AGC Ser	ATC Ile	777
CTT Leu 220	ACA Thr	TCG Ser	TTG Leu	GGT Gly	TCA Ser 225	ACC Thr	CCC Pro	ATT Ile	ATT Ile	GAA Glu 230	CAA Gln	AGT Ser	ACC Thr	AAG Lys	GGT Gly 235	825
GGC Gly	ATC Ile	TCT Ser	CTT Leu 240	CCA Pro	ATT Ile	GGT Gly	CTG Leu	ATT Ile	GTT Val 245	GGA Gly	GTG Val	ACA Thr	TCA Ser	CTG Leu 250	GGT Gly	873
CTG Leu	CTG Leu	ATG Met 255	TTA Leu	GGA Gly	CTG Leu	GTG Val	AAC Asn 260	TGC Cys 260	ATC Ile	ATC Ile	CTG Leu	GTG Val	CAG Gln 265	AGG Arg	AAA Lys	921
AAG Lys	AAG Lys	CCC Pro 270	TCC Ser	TGC Cys	CTA Leu	CAA Gln	AGA Arg 275	GAT Asp	GCC Ala	AAG Lys	GTG Val	CCT Pro 280	CAT His	GTG Val	CCT Pro	969
GAT Asp	GAG Glu 285	AAA Lys	TCC Ser	CAG Gln	GAT Asp	GCA Ala 290	GTA Val	GGC Gly	CTT Leu	GAG Glu	CAG Gln 295	CAG Gln	CAC His	CTG Leu	TTG Leu	1017
ACC Thr 300	ACA Thr	GCA Ala	CCC Pro	AGT Ser	TCC Ser 305	AGC Ser	AGC Ser	AGC Ser	TCC Ser	CTA Leu 310	GAG Glu	AGC Ser	TCA Ser	GCC Ala	AGC Ser 315	1065
GCT Ala	GGG Gly	GAC Asp	CGA Arg 320	AGG Arg	GCG Ala	CCC Pro	CCT Pro	GGG Gly	GGC Gly 325	CAT His	CCC Pro	CAA Gln	GCA Ala	AGA Arg 330	GTC Val	1113
ATG Met	GCG Ala	GAG Glu	GCC Ala 335	CAA Gln	GGG Gly	TTT Phe	CAG Gln	GAG Glu 340	GCC Ala	CGT Arg	GCC Ala	AGC Ser	TCC Ser 345	AGG Arg	ATT Ile	1161
TCA Ser	GAT Asp	TCT Ser 350	TCC Ser	CAC His	GGA Gly	AGC Ser	CAC His 355	GGG Gly	ACC Thr	CAC His	GTC Val	AAC Asn 360	GTC Val	ACC Thr	TGC Cys	1209
ATC Ile	GTG Val 365	AAC Asn	GTC Val	TGT Cys	AGC Ser	AGC Ser 370	TCT Ser	GAC Asp	CAC His	AGT Ser	TCT Ser 375	CAG Gln	TGC Cys	TCT Ser	TCC Ser	1257
CAA Gln 380	GCC Ala	AGC Ser	GCC Ala	ACA Thr	GTG Val 385	GGA Gly	GAC Asp	CCA Pro	GAT Asp	GCC Ala 390	AAG Lys	CCC Pro	TCA Ser	GCG Ala	TCC Ser 395	1305

CCA AAG GAT GAG CAG GTC CCC TTC TCT CAG GAG GAG TGT CCG TCT CAG	1353
Pro Lys Asp Glu Gln Val Pro Phe Ser Gln Glu Glu Cys Pro Ser Gln	
400 405 410	
TCC CCG TGT GAG ACT ACA GAG ACA CTG CAG AGC CAT GAG AAG CCC TTG	1401
Ser Pro Cys Glu Thr Thr Glu Thr Leu Gln Ser His Glu Lys Pro Leu	
415 420 425	
CCC CTT GGT GTG CCG GAT ATG GGC ATG AAG CCC AGC CAA GCT GGC TGG	1449
Pro Leu Gly Val Pro Asp Met Gly Met Lys Pro Ser Gln Ala Gly Trp	
430 435 440	
TTT GAT CAG ATT GCA GTC AAA GTG GCC TGACCCCTGA CAGGGGTAAC	1496
Phe Asp Gln Ile Ala Val Lys Val Ala	
445 450	
ACCCTGCAAA GGGACCCCCG AGACCCTGAA CCCATGGAAC TTCATGACTT TTGCTGGATC	1556
CATTTCCCTT AGTGGCTTCC AGAGCCCCAG TTGCAGGTCA AGTGAGGGCT GAGACAGCTA	1616
GAGTGGTCAA AAAGTGCCAT GGTGTTTTAT GGGGGCAGTC CCAGGAAGTT GTTGCTCTTC	1676
CATGACCCCT CTGGATCTCC TGGGCTCTTG CCTGATTCTT GCTTCTGAGA GGCCCCAGTA	1736
TTTTTTCCTT CTAAGGAGCT AACATCCTCT TCCATGAATA GCACAGCTCT TCAGCCTGAA	1796
TGCTGACACT GCAGGGCGGT TCCAGCAAGT AGGAGCAAGT GGTGGCCTGG TAGGGCACAG	1856
AGGCCCTTCA GGTTAGTGCT AAAGTCTTAG GAAGTACCCT CTCCAAGCCC ACCGAAATTC	1916
TTTTGATGCA AGAATCAGAG GCCCCATCAG GCAGAGTTGC TCTGTTATAG GATGGTAGGG	1976
CTGTAACTCA GTGGTCCAGT GTGCTTTTAG CATGCCCTGG GTTTGATCCT CAGCAACACA	2036
TGCAAAACGT AAGTAGACAG CAGACAGCAG ACAGCACAGC CAGCCCCCTG TGTGGTTTGC	2096
AGCCTCTGCC TTTGACTTTT ACTCTGGTGG GCACACAGAG GGCTGGAGCT CCTCCTCCTG	2156
ACCTTCTAAT GAGCCCTTCC AAGGCCACGC CTTCTTCAG GGAATCTCAG GGACTGTAGA	2216
GTTCCCAGGC CCCTGCAGCC ACCTGTCTCT TCCTACCTCA GCCTGGAGCA CTCCCTCTAA	2276
CTCCCCAACG GCTTGGTACT GTACTTGCTG TGACCCCAAC GTGCATTGTC CGGGTTAGGC	2336
ACTGTGAGTT GGAACAGCTC ATGACATCGG TTGAAAGGCC CACCCGAAA CAGCTAAGCC	2396
AGCTCTTTTG CCAAAGGATT CATGCCGGTT TTCTAATCAA CCTGCTCCCT AGCATTGCCT	2456
GGAAGGAAAG GGTTCAAGAG ACTCCTCAAG AAGCAAGTTC AGTCTCAGGT GCTTGATGTC	2516
CATGCTCACC GATTCCACTG GATATGAACT TGGCAGAGGA GCCTAGTTGT TGCCATGGAG	2576
ACTTAAAGAG CTCAGCACTC TGGAATCAAG ATACTGGACA CTTGGGGCCG ACTTGTTAAG	2636
GCTCTGCAGC ATCAGACTGT AGAGGGGAAG GAACACGTCT GCCCCCTGGT GGCCCGTCCT	2696

GGGATGACCT CGGGCCTCCT AGGCAACAAA AGAATGAATT GGAAAGGATG TTCCTGGGTG	2756
TGGCCTAGCT CCTGTGCTTG TGTGGATCCC TAAAGGGTGT GCTAAGGAGC AATTGCACTG	2816
TGTGCTGGAC AGAATTCCTG CTTATAAATG CTTTTTGTGTTG TTGTTTTGTA CACTGAGCCC	2876
TGGCTGAGCC ACCCCACCCC ACCTCCCATC CCACCTTTAC ACGCCACTCT TGCATGAGAA	2936
CCTGGCTGTC TCCCACCTTG AGCCTGTGGA TGCTGAGGAA ACACCCAGCC AAGTAGACTC	2996
CAGGCTTGCC CCTATCTCCT GCTATGAGTC TGGCCTCCTC ATTGTGTTGT GGGAAGGAGA	3056
CGGGTTCTGT CATCTCGGAA CGCCACACCC GTGGATGTGA ACAATGGCTG TACTAGCTTA	3116
GACCAGCTTA GGGCTCTGCA TATCACAGGA GGGGGAGCAG GGAACAATTT GAGTGCTGAC	3176
CTATAACACA GTTCCTAAAG GATCGGGCAG TCCAGAATCT CCTCCTTCAG TGTGTGTGTG	3236
TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTCCATGTT TGCATGTATG TGTGTGCCAG	3296
TGTGTGGAGG CCCGAGGTTG GCTTTGGGTG TGTTTGATCA CTCTCCAGTT ACTGAGGCGG	3356
GCTCTCATCT GTACCCAGAG CTTGCACATT TTCTAGTCTA ACTTGATTCA GGGATCTCTG	3416
TCTGCCTATG GAGGTGCTCA GGTTACAGGC AGGCTGCCAT ACCTGCCCCGA CATTTACATG	3476
AATACTAGAG ATCTGAATTC TGGTCCTCAC ACTTGTATAC CTGCATTTTA TCCACTAAGA	3536
CATCTCTCCA AGGGCTCCCC CTTCTATTTT AATAAGTTAG TTTTGAACTG GCAAGATGGC	3596
TCAGTGGGTA AGGCAGTTTG CGGACAAACC TGATGACCTG AGTTGGATCC CTGACCATAA	3656
GGTAGAAGAG ACCTGATTCC TGCAAGTTGT CCTCTGACCA CCACCCCATTA CATGCTTCTG	3716
CATATGTGCA CACATCACAT TCTTGACAC ACACCTCACAT ACCATAAATG TAATAAATTT	3776
TTTTAAATAA ATTGATTTTA TCTTTTAAAA AAAAAA	3813

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 474 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Ala	Pro	Ala	Ala	Leu	Trp	Val	Ala	Leu	Val	Phe	Glu	Leu	Gln	Leu
-22		-20					-15.					-10			
Trp	Ala	Thr	Gly	His	Thr	Val	Pro	Ala	Gln	Val	Val	Leu	Thr	Pro	Tyr
-5						1				5					10

Lys Pro Glu Pro Gly Tyr Glu Cys Gln Ile Ser Gln Glu Tyr Tyr Asp
 15 20 25
 Arg Lys Ala Gln Met Cys Cys Ala Lys Cys Pro Pro Gly Gln Tyr Val
 30 35 40
 Lys His Phe Cys Asn Lys Thr Ser Asp Thr Val Cys Ala Asp Cys Glu
 45 50 55
 Ala Ser Met Tyr Thr Gln Val Trp Asn Gln Phe Arg Thr Cys Leu Ser
 60 65 70
 Cys Ser Ser Ser Cys Thr Thr Asp Gln Val Glu Ile Arg Ala Cys Thr
 75 80 85 90
 Lys Gln Gln Asn Arg Val Cys Ala Cys Glu Ala Gly Arg Tyr Cys Ala
 95 100 105
 Leu Lys Thr His Ser Gly Ser Cys Arg Gln Cys Met Arg Leu Ser Lys
 110 115 120
 Cys Gly Pro Gly Phe Gly Val Ala Ser Ser Arg Ala Pro Asn Gly Asn
 125 130 135
 Val Leu Cys Lys Ala Cys Ala Pro Gly Thr Phe Ser Asp Thr Thr Ser
 140 145 150
 Ser Thr Asp Val Cys Arg Pro His Arg Ile Cys Ser Ile Leu Ala Ile
 155 160 165 170
 Pro Gly Asn Ala Ser Thr Asp Ala Val Cys Ala Pro Glu Ser Pro Thr
 175 180 185
 Leu Ser Ala Ile Pro Arg Thr Leu Tyr Val Ser Gln Pro Glu Pro Thr
 190 195 200
 Arg Ser Gln Pro Leu Asp Gln Glu Pro Gly Pro Ser Gln Thr Pro Ser
 205 210 215
 Ile Leu Thr Ser Leu Gly Ser Thr Pro Ile Ile Glu Gln Ser Thr Lys
 220 225 230
 Gly Gly Ile Ser Leu Pro Ile Gly Leu Ile Val Gly Val Thr Ser Leu
 235 240 245 250
 Gly Leu Leu Met Leu Gly Leu Val Asn Cys Ile Ile Leu Val Gln Arg
 255 260 265
 Lys Lys Lys Pro Ser Cys Leu Gln Arg Asp Ala Lys Val Pro His Val
 270 275 280
 Pro Asp Glu Lys Ser Gln Asp Ala Val Gly Leu Glu Gln Gln His Leu
 285 290 295

Leu	Thr	Thr	Ala	Pro	Ser	Ser	Ser	Ser	Ser	Ser	Leu	Glu	Ser	Ser	Ala
300						305					310				
Ser	Ala	Gly	Asp	Arg	Arg	Ala	Pro	Pro	Gly	Gly	His	Pro	Gln	Ala	Arg
315					320					325					330
Val	Met	Ala	Glu	Ala	Gln	Gly	Phe	Gln	Glu	Ala	Arg	Ala	Ser	Ser	Arg
				335					340					345	
Ile	Ser	Asp	Ser	Ser	His	Gly	Ser	His	Gly	Thr	His	Val	Asn	Val	Thr
			350					355					360		
Cys	Ile	Val	Asn	Val	Cys	Ser	Ser	Ser	Asp	His	Ser	Ser	Gln	Cys	Ser
		365					370					375			
Ser	Gln	Ala	Ser	Ala	Thr	Val	Gly	Asp	Pro	Asp	Ala	Lys	Pro	Ser	Ala
		380				385					390				
Ser	Pro	Lys	Asp	Glu	Gln	Val	Pro	Phe	Ser	Gln	Glu	Glu	Cys	Pro	Ser
395					400					405					410
Gln	Ser	Pro	Cys	Glu	Thr	Thr	Glu	Thr	Leu	Gln	Ser	His	Glu	Lys	Pro
				415					420					425	
Leu	Pro	Leu	Gly	Val	Pro	Asp	Met	Gly	Met	Lys	Pro	Ser	Gln	Ala	Gly
			430					435					440		
Trp	Phe	Asp	Gln	Ile	Ala	Val	Lys	Val	Ala						
		445					450								